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(54) Title: ADENOVIRUS DERIVED GENE DELIVERY VEHICLES COMPRISING AT LEAST ONE ELEMENT OF ADENOVIRUS TYPE 35

(57) Abstract

The serotypes differ in their natural tropism. The adenovirus serotypes (2, 4, 5 and 7) all have a natural affiliation towards lung epithelia and other respiratory tissues. In contrast, serotypes (40 and 41) have a natural affiliation towards the gastrointestinal tract. The serotypes described above, differ in at least capsid proteins (penton–base, hexon), proteins responsible for cell binding (fiber protein), and proteins involved in adenovirus replication. This difference in tropism and capsid protein among serotypes has led to the many research efforts aimed at redirecting the adenovirus tropism by modification of the capsid proteins.

ADENOVIRUS DERIVED GENE DELIVERY VEHICLES COMPRISING
AT LEAST ONE ELEMENT OF ADENOVIRUS TYPE 35

The present invention relates to the field of gene therapy, in particular gene therapy involving elements derived from viruses, more in particular elements of adenoviruses. Adenoviruses have been proposed as suitable vehicles to

5   deliver genes to the host.

There are a number of features of adenoviruses that make them particularly useful for the development of gene-transfer vectors for human gene therapy:

The adenovirus genome is well characterized. It consists of

10   a linear double-stranded DNA molecule of approximately 36000 base pairs. The adenovirus DNA contains identical Inverted Terminal Repeats (ITR) of approximately 90-140 base pairs with the exact length depending on the serotype. The viral origins of replication are within the ITRs exactly at the

15   genome ends;

The biology of the adenoviruses is characterized in detail; the adenovirus is not associated with severe human pathology in immuno-competent individuals

The virus is extremely efficient in introducing its DNA into

20   the host cell; the virus can infect a wide variety of cells and has a broad host-range;

The virus can be produced at high virus titers in large quantities;

The virus can be rendered replication defective by deletion

25   of the early-region 1 (E1) of the viral genome (Brody et al, 1994). Most adenoviral vectors currently used in gene therapy have a deletion in the E1 region, where desired genetic information can be introduced.

30   Based on these features, preferred methods for in vivo gene transfer into human target cells, make use of adenoviral vectors as gene delivery vehicles.

However, there are still drawbacks associated with the therapeutic use of adenoviral vectors in humans. A major

drawback is the existence of widespread pre-existing
immunity among the population against adenoviruses. Exposure
to wild-type adenoviruses is very common in humans, as has
been documented extensively [reviewed in Wadell, 1984]. This
5   exposure has resulted in immune responses against most types
of adenoviruses, not alone against adenoviruses to which
individuals have actually been exposed, but also against
adenoviruses which have similar (neutralizing) epitopes.
This phenomenon of pre-existing antibodies in humans, in
10 combination with a strong secondary humoral and cellular
immune response against the virus, can seriously affect gene
transfer using recombinant adenoviral vectors.
To date, six different subgroups of human adenoviruses have
been proposed which in total encompasses 51 distinct
15 adenovirus serotypes (see table 1). A serotype is defined on
the basis of its immunological distinctiveness as determined
by quantitative neutralization with animal antisera (horse,
rabbit). If neutralization shows a certain degree of cross-
reaction between two viruses, distinctiveness of serotype is
20 assumed if A) the hemagglutinins are unrelated, as shown by
lack of cross-reaction on hemagglutination-inhibition, or B)
substantial biophysical/ biochemical differences in DNA
exist (Francki et al, 1991). The nine serotypes identified
last (42-51) were isolated for the first time from HIV-
25 infected patients (Hierholzer et al 1988; Schnurr et al
1993;). For reasons not well understood, most of such
immune-compromised patients shed adenoviruses that were
rarely or never isolated from immune-competent individuals
(Hierholzer et al 1988, 1992; Khoo et al, 1995, De Jong et
30 al, 1998).
The vast majority of individuals have had previous exposure
to adenoviruses, especially the well investigated adenovirus
serotypes 5 and type 2 (Ad5 and Ad2) or immunologically
related serotypes. Importantly, these two serotypes are also
35 the most extensively studied for use in human gene therapy.

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from other (adeno) viruses, as long as one replaces an
element which could lead to immunity against such a gene
delivery vehicle by an element of adenovirus 35 or a
functional homologue thereof, which has less of such a
5   drawback and which preferably avoids such a drawback. In the
present invention a gene delivery vehicle is any vehicle
that is capable of delivering a nucleic acid of interest to
a host cell. It must, according to the invention comprise an
element of adenovirus 35 or a functional equivalent of such
10   an element, which must have a beneficial effect regarding
the immune response against such a vehicle. Basically all
other elements making up the vehicle can be any elements
known in the art or developed in the art, as long as
together they are capable of delivering said nucleic acid of
15   interest. In principle the person skilled in the art can use
and/or produce any adenoviral products or production systems
that can or have been applied in the adenoviral field.
Typically the products of the invention can be made in the
packaging cells useable for e.g. adenovirus 5, typically the
20   vectors based on adenovirus 35 can be produced and/or used
in the same manner as those of other adenoviruses e.g.
adenovirus 2 and/or 5. A good overview of the possibilities
of minimal vectors, packaging systems, intracellular
amplification, vector and plasmid based systems can be found
25   in applicant's copending applications (PCT/NL99/00235 and
PCT/NL96/00244) incorporated herein by reference. Non-viral
delivery systems can also be provided with elements
according to the invention as can viral delivery systems.
Both kinds of systems are well known in the art in many
30   different set-ups and do therefor not need any further
elaboration here. A review on the many different systems and
their properties can be found in Robbins and Ghivizzani
(1998) and in Prince (1998) incorporated herein by
reference.
35   Gene delivery vehicles typically contain a nucleic acid of
interest. A nucleic acid of interest can be a gene or a

functional part of a gene (wherein a gene is any nucleic
acid which can be expressed) or a precursor of a gene or a
transcribed gene on any nucleic acid level (DNA and/or RNA:
double or single stranded). Genes of interest are well known
5   in the art and typically include those encoding therapeutic
proteins such as TPA, EPO, cytokines, antibodies or
derivatives thereof, etc. An overview of therapeutic
proteins to be applied in gene therapy are listed
below.
10   Immune-stimulatory factors like tumor-specific antigens,
cytokines, etc.;
Anti-angiogenic factors non-limiting examples endostatin,
angiostatin, ATF-BPTI CDT-6, dominant negative VEGF-mutants,
etc.;
15   Angiogenic factors non-limiting example VEGF, Fibroblast
growth factors, Nitric oxide synthases, C-type natriuretic
peptide, etc.;
Inflammation inhibiting proteins like soluble CD40, FasL,
IL-12, IL-10, IL-4, IL-13 and excreted single chain
20   antibodies to CD4, CD5, CD7, CD52, Il-2, IL-1, IL-6, TNF,
etc. or excreted single chain antibodies to the T-cell
receptor on the auto-reactive T-cells. Also, dominant
negative mutants of PML may be used to inhibit the immune
response.
25   Furthermore, antagonists of inflammation promoting cytokines
may be used, for example IL-1RA(receptor antagonist) and
soluble receptors like sIL-1RI, sIL-1RII, sTNFRI and
sTNFRII. Growth and/or immune response inhibiting genes such
as ceNOS, Bcl3, cactus and IκBα, β or γ and apoptosis
30   inducing proteins like the VP3 protein of chicken anemia
virus may also be used. Furthermore, suicide genes like HSV-
TK, cytosine deaminase, nitroreductase and linamerase may be
used.

35   A nucleic acid of interest may also be a nucleic acid which
can hybridise with a nucleic acid sequence present in the

host cell thereby inhibiting expression or transcription or translation of said nucleic acid. It may also block through cosuppression. In short a nucleic acid of interest is any nucleic acid that one may wish to provide a cell with in

5   order to induce a response by that cell, which response may be production of a protein, inhibition of such production, apoptosis, necrosis, proliferation, differentation etc. The present invention is the first to disclose adenovirus 35 or a functional homologue thereof for therapeutical use,

10   therefor the invention also provides an adenovirus serotype 35 or a functional homologue thereof or a chimaeric virus derived therefrom, or a gene delivery vehicle based on said virus its homologue or its chimaera for use as a pharmaceutical. The serotype of the present invention,

15   adenovirus type 35, is in itself known in the art. It is an uncommon group B adenovirus that was isolated from patients with acquired immunodeficiency syndrome ana other immunodeficiency disorders (Flomenberg et al., 1987; De Jong et al., 1983). Ad 35 has been shown to differ from the more

20   fully characterized subgroup C (including Ad2 and Ad5) with respect to pathogenic properties (Basler et al., 1996). It has been suggested that this difference may be correlated with differences in the E3 region of the Ad35 genome (Basler et al., 1996). The DNA of Ad35 has been partially cloned and

25   mapped (Kang et al., 1989a and b; Valderrama-Leon et al., 1985). B type adenovirus serotypes such as 34 and 35 have a different E3 region than other serotypes. Typically this region is involved in suppressing immune response to

30   adenoviral products. Thus the invention provides a gene delivery vehicle according to the invention whereby said elements involved in avoiding or diminishing immune response comprise adenovirus 35 E3 expression products or the genes encoding them or functional equivalents of either or both.

Another part of adenoviruses involved in immune responses is
the capsid, in particular the penton and/or the hexon
proteins. Thus the invention also provides a gene delivery
vehicle according to the invention whereby the elements
5   comprise at least one adenovirus 35 capsid protein or
functional part thereof, such as fiber, penton and/or hexon
proteins or a gene encoding at least one of them. It is not
necessary that a whole protein relevant for immune response
is of adenovirus 35 (or a functional homologue thereof)
10   origin. It is very well possible to insert a part of an
adenovirus fiber, penton or hexon protein into another
fiber, penton or hexon. Thus chimaeric proteins are
obtained.
It is also possible to have a penton of a certain
15   adenovirus, a hexon from another and a fiber or an E3 region
from yet another adenovirus. According to the invention at
least one of the proteins or genes encoding them should
comprise an element from adenovirus 35 or a functional
homologue thereof, whereby said element has an effect on the
20   immune response of the host. Thus the invention provides a
gene delivery according to the invention, which is a
chimaera of adenovirus 35 with at least one other
adenovirus. In this way one can also modify the resulting
virus in other aspects then the immune response alone. One
25   can enhance its efficiency of infection with elements
responsible therefor; one can enhance its replication on a
packaging cell, or one can change its tropism.
Thus the invention e.g. provides a gene delivery vehicle
according to the invention which has a different tropism
30   than adenovirus 35. Of course the tropism should be altered
preferably such that the gene delivery vehicle is delivered
preferentially to a subset of the host's cells, i.e. the
target cells. Changes in tropism and other changes which can
also be applied in the present invention of adenoviral or
35   other gene delivery vehicles are disclosed in applicant's
copending applications (nos. 98204482.8, 99200624.7 and

98202297.2) incorporated herein by reference. Of course the
present application also provides any and all building
blocks necessary and/or useful to get to the gene delivery
vehicles and/or the chimaeras, etc. of the present

5   invention. This includes packaging cells such as PER.C6
(ECACC deposit number 96022940) or cells based thereon, but
adapted for Ad35 or a functional homologue thereof; it also
includes any nucleic acids encoding functional parts of
adenovirus 35 or a functional homologue thereof, such as

10   helper constructs and packaging constructs, as well as
vectors comprising genes of interest and e.g. an ITR, etc.
Typically applicant's application (PCT/NL96/00244)
incorporated herein by reference, discloses elements
necessary and useful for arriving at the invented gene

15   delivery vehicles. Thus the invention also provides a
nucleic acid encoding at least a functional part of a gene
delivery vehicle according to the invention, or a virus,
homologue or chimaera thereof according to the invention.
According to the invention, such elements, which encode

20   functions that will end up in the resulting gene delivery
vehicle must comprise or be encoded by a nucleic acid
encoding at least one of the adenovirus serotype 35 elements
or a functional equivalent thereof, responsible for avoiding
or deminishing neutralising activity against adenoviral

25   elements by the host to which the gene is to be delivered.
Typically the gene of interest would be present on the same
nucleic acid which means that such a nucleic acid has such a
gene or that it has a site for introducing a gene of
interest therein.

30   Typically such a nucleic acid also comprises at least one
ITR and if it is a nucleic acid to be packaged also a
packaging signal. However, as mentioned before all necessary
and useful elements and/or building blocks for the present
invention can be found in applicant's application

35   (PCT/NL96/00244). A set of further improvements in the field
of producing adenoviral gene delivery vehicles is

applicant's plasmid system disclosed in PCT/NL99/00235
mentioned herein before. This system works in one embodiment
as a homologous recombination of an adapter plasmid and a
longer plasmid, together comprising all elements of the

5   nucleic acid to be incorporated in the gene delivery
vehicle. These methods can also be applied to the presently
invented gene delivery vehicles and their building elements.
Thus the invention also provides a nucleic acid according
to the invention further comprising a region of nucleotides

10   designed or useable for homologous recombination, preferably
as part of at least one set of two nucleic acids comprising
a nucleic acid according to the invention, whereby said set
of nucleic acids is capable of a single homologous
recombination event with each other, which leads to a

15   nucleic acid encoding a functional gene delivery vehicle.
Both empty packaging cells (in which the vector to be
packaged to make a gene delivery vehicle according to the
invention still has to be introduced or produced) as well as
cells comprising a vector according to the invention to be

20   packaged are provided. Thus the invention also encompasses a
cell comprising a nucleic acid according to the invention or
a set of nucleic acids according to the invention,
preferably a cell which complements the necessary elements
for adenoviral replication which are absent from the nucleic

25   acid according to be packaged, or from a set of nucleic
acids according to the invention. In the present invention
it has been found that E1-deleted adenovirus 35 vectors, are
not capable of replication on cells that provide adenovirus
5 proteins in trans. The invention therefore further

30   provides a cell capable of providing adenovirus 35 E1
proteins in trans. Such a cell is typically a human cell
derived from the retina or the kidney. Embryonal cells such
as amniocytes, have been shown to be particularly suited for
the generation of an E1 complementing cell line. Such cells

35   are therefor preferred in the present invention. Serotype
specific complementation by E1 proteins can be due to one or

more protein(s) encoded by the E1 region. It is therefor
essential that at least the serotype specific protein is
provided in trans in the complementing cell line. The non-
serotype specific E1 proteins essential for effective

5   complementation of an E1-deleted adenovirus can be derived
from other adenovirus serotpyes. Preferably, at least an E1
protein from the E1B region of adenovirus 35 is provided in
trans to complement E1-deleted adenovirus 35 based vectors.
In one embodiment nucleic acid encoding the one or more

10   serotype specific E1-proteins is introduced into the PER.C6
cell or a cell originating from a PER.C6 cell (ECACC deposit
number 96022940), or a similar packaging cell complementing
with elements from Ad 35 or a functional homologue thereof.
As already alluded to the invention also encompasses a

15   method for producing a gene delivery vehicle according to
the invention, comprising expressing a nucleic acid
according to the invention in a cell according to the
invention and harvesting the resulting gene delivery
vehicle. The above refers to the filling of the empty

20   packaging cell with the relevant nucleic acids. The format
of the filled cell is of course also part of the present
invention, which provides a method for producing a gene
delivery vehicle according to the invention, comprising
culturing a filled packaging cell (producer cell) according

25   to the invention in a suitable culture medium and harvesting
the resulting gene delivery vehicle.
The resulting gene delivery vehicles obtainable by any
method according to the invention are of course also part of
the present invention, particularly also a gene delivery

30   vehicle according to the invention, which is derived from a
chimaera of an adenovirus and an integrating virus.
It is well known that adenoviral gene delivery vehicles do
not integrate into the host genome normally. For long term
expression of genes in a host cell it is therefor preferred

35   to prepare chimaeras which do have that capability. Such
chimaeras have been disclosed in our copending application

PCT/NL98/00731 incorporated herein by reference. A very good
example of such a chimaera of an adenovirus and an
integrating virus wherein said integrating virus is an adeno
associated virus. As discussed hereinbefore other useful

5   chimaeras, which can also be combined with the above are
chimaeras (be it in swapping whole proteins or parts thereof
or both) which have altered tropism. A very good example
thereof is a chimaera of Ad 35 and Ad 16, possibly with
elements from for instance Ad 2 or Ad 5, wherein the tropism

10   determining part of Ad 16 or a functional equivalent thereof
is used to direct the gene delivery vehicle to synoviocytes
and/or smooth muscle cells (see our copending applications
nos. 98204482.8 and 99200624.7) incorporated herein by
reference). Dendritic cells (DC) and hemopoietic stem cells

15   (HSC) are not easily transduced with Ad2 or Ad5 derived gene
delivery vehicles. The present invention provides gene
aelivery vehicles chac posses increaseu cransduccion
capacity of DC and HSC cells. Such gene delivery vehicles at
least comprises the tissue tropism determining part of an

20   Ad35 adenovirus. The invention therefore further provides
the use of a tissue tropism determining part of an
adenovirus 35 capsid for transducing dendritic cells and/or
hemopoietic stem cells. Other B-type adenoviruses are also
suited. A tissue tropism determining part comprises at least

25   the knob and/or the shaft of a fiber protein. Of course it
is very well possible for a person skilled in the art to
determine the amino acid sequences responsible for the
tissue tropism in the fiber protein. Such knowledge can be
used to devise chimearic proteins comprising such amino acid

30   sequences. Such chimaeric proteins are therefor also part of
the invention. DC cells are very efficient antigen
presenting cells. By introducing the gene delivery vehicle
into such cells the immune system of the host can be
triggered to toward specific antigens. Such antigens can be

35   encoded by nucleic acid delivered to the DC or by the
proteins of the gene delivery vehicle it self. The present

invention therefor also provides a gene delivery vehicle
with the capacity to evade to host immune system as a
vaccine. The vector being capable to evade the immune system
long enough to efficiently find it target cells and at the

5   same time capable of delivering specific antigens to antigen
presenting cells thereby allowing the induction and/or
stimulation of an efficient immune responses toward the
specific antigen(s). To further modulate the immune
response, the gene delivery vehicle may comprise proteins

10   and/or nucleic encoding such proteins capable of modulating
an immune response. Non-limiting examples of such proteins
are found among the interleukins, the adhesion molecules,
the co-stimulatory proteins, the interferons etc. The
invention therefore further provides a vaccine comprising a

15   gene delivery vehicle of the invention. The invention
further provides an adenovirus vector with the capacity to
efficiently transduce DC and/or HSC, the vehicle comprising
at least a tissue tropism determing part of serotype 35
adenvirus. The invention further provides the use of such

20   delivery vehicles for the transduction of HSC and/or DC
cells. Similar tissue tropisms are found among other
adenoviruses of serotype B, particularly in serotype 11 and
are also part of the invention. Of course it is also
possible to provide other gene delivery vehicles with the

25   tissue tropism determining part thereby providing such
delivery vehicles with an enhanced DC and/or HSC
transduction capacity. Such gene delivery vehicles are
therefor also part of the invention.
The gene delivery vehicles according to the invention can be

30   used to deliver genes or nucleic acids of interest to host
cells. This will typically be a pharmaceutical use. Such a
use is included in the present invention. Compositions
suitable for such a use are also part of the present
invention. The amount of gene delivery vehicle that needs to

35   be present per dose or per infection (m.o.i) will depend on
the condition to be treated, the route of administration

(typically parenteral) the subject and the efficiency of
infection, etc. Dose finding studies are well known in the
art and those already performed with other (adenoviral) gene
delivery vehicles can typically be used as guides to find
5   suitable doses of the gene delivery vehicles according to
the invention. Typically this is also where one can find
suitable excipients, suitable means of administration,
suitable means of preventing infection with the vehicle
where it is not desired, etc. Thus the invention also
10   provides a pharmaceutical formulation comprising a gene
delivery vehicle according to the invention and a suitable
excipient, as well as a pharmaceutical formulation
comprising an adenovirus, a chimaera thereof, or a
functional homologue thereof according to the invention and
15   a suitable excipient.

Detailed description

As described above, the most extensively studied serotypes
20   of adenovirus are not ideally suitable for delivering
additional genetic material to host cells. This is partly
due to the pre-existing immunity among the population
against these serotypes. This presence of pre-existing
antibodies in humans, in combination with a strong secondary
25   humoral and cellular immune response against the virus will
affect adenoviral gene therapy.

The present invention provides the use of at least elements
of a serotype and functional homologues thereof of
30   adenovirus which are very suitable as gene therapy vectors.
The present invention also discloses an automated high-
throughput screening of all known adenovirus serotypes
against sera from many individuals. Surprisingly, no
neutralizing ability was found in any of the sera that were
35   evaluated against one particular serotype, adenovirus 35
(Ad35). This makes the serotype of the present invention

extremely useful as a vector system for gene therapy in man.
Such vector system is capable of efficiently transferring
genetic material to a human cell without the inherent
problem of pre-exisiting immunity.

5   Typically, a virus is produced using an adenoviral vector
(typically a plasmid, a cosmid or baculovirus vector). Such
vectors are of course also part of the present invention.
The invention also provides adenovirus derived vectors that
have been rendered replication defective by deletion or

10   inactivation of the E1 region. Of course, also a gene of
interest can be inserted at for instance the site of E1 of
the original adenovirus from which the vector is derived.
In all aspects of the invention the adenoviruses may contain
deletions in the E1 region and insertions of heterologous

15   genes linked either or not to a promoter. Furthermore, the
adenoviruses may contain deletions in the E2, E3 or E4
regions and insertions of heterologous genes linked to a
promoter. In these cases, E2 and/or E4 complementing cell
lines are required to generate recombinant adenoviruses.

20

One may choose to use the Ad35 serotype itself for the
preparation of recombinant adenoviruses to be used in gene
therapy. Alternatively, one may choose to use elements
derived from the serotype of the present invention in such

25   recombinant adenoviruses. One may for instance develop a
chimaeric adenovirus that combines desirable properties from
different serotypes. Some serotypes have a somewhat limited
host range, but have the benefit of being less immunogenic,
some are the other way round. Some have a problem of being

30   of a limited virulence, but have a broad host range and/or a
reduced immunogenicity. Such chimaeric adenoviruses are
known in the art, and they are intended to be within the
scope of the present invention. Thus in one embodiment the
invention provides a chimaeric adenovirus comprising at

35   least a part of the adenovirus genome of the present
serotype, providing it with absence of pre-existing

immunity, and at least a part of the adenovirus genome from
another adenovirus serotype resulting in a chimaeric
adenovirus. In this manner the chimaeric adenovirus produced
is such that it combines the absence of pre-existing
5   immunity of the serotype of the present invention, to other
characteristics of another serotype. Such characteristics
may be temperature stability, assembly, anchoring,
redirected infection, production yield, redirected or
improved infection, stability of the DNA in the target cell,
10   etc.
A packaging cell will generally be needed in order to
produce sufficient amount of adenoviruses. For the
production of recombinant adenoviruses for gene therapy
purposes, several cell lines are available. These include
15   but are not limited to the known cell lines PER.C6 (ECACC
deposit number 96022940), 911, 293, and El A549.
An important feature of the present invention is the means
to produce the adenovirus. Typically, one does not want an
adenovirus batch for clinical applications to contain
20   replication competent adenovirus. In general therefore, it
is desired to omit a number of genes (but at least one) from
the adenoviral genome on the adenoviral vector and to supply
these genes in the genome of the cell in which the vector is
brought to produce chimaeric adenovirus. Such a cell is
25   usually called a packaging cell. The invention thus also
provides a packaging cell for producing an adenovirus (a
gene delivery vehicle) according to the invention,
comprising in trans all elements necessary for adenovirus
production not present on the adenoviral vector according to
30   the invention. Typically vector and packaging cell have to
be adapted to one another in that they have all the
necessary elements, but that they do not have overlapping
elements which lead to replication competent virus by
recombination.
35

Thus the invention also provides a kit of parts comprising a packaging cell according to the invention and a recombinant vector according the invention whereby there is essentially no sequence overlap leading to recombination resulting in

5   the production of replication competent adenovirus between said cell and said vector.

Thus the invention provides methods for producing adenovirus, which upon application will escape pre-existing

10   humoral immunity, comprising providing a vector with elements derived from an adenovirus serotype against which virtually no natural immunity exists and transfecting said vector in a packaging cell according to the invention and allowing for production of viral particles.

15

In one aspect this invention describes the use of the adenovirus serotype of the present invention to overcome natural existing or induced, neutralising host activity towards adenoviruses administered in vivo for therapeutic

20   applications. The need for a new serotype is stressed by observations that 1) repeated systemic delivery of recombinant adenovirus serotype 5 is unsuccessful due to formation of high titers of neutralising antibodies against the recombinant adenovirus serotype 5 (Schulick et al,

25   1997), and 2) pre-existing or humoral immunity is widespread in the population.
In another aspect this invention provides the use of gene delivery vehicles of the invention or the use of adenovirus serotype 35 for vaccination purposes. Such use prevents at

30   least in part undesired immune responses of the host. Non-limiting examples of undesired immune responses are evoking an immune response against the gene delivery vehicle or adenovirus serotype 35 and/or boosting of an immune response against the gene delivery vehicle or adenovirus serotype 35.

35   · In another aspect of the invention, alternating use is made of Ad vectors belonging to different subgroups. This aspect

of the invention therefore circumvents the inability to
repeat the administration of an adenovirus for gene therapy
purposes.

Example 1

A high throughput assay for the detection of neutralising activity in human serum

5

To enable screening of a large amount of human sera for the presence of neutralising antibodies against all adenovirus serotypes, an automated 96-wells assay was developed.
Human sera

10   A panel of 100 individuals was selected. Volunteers (50% male, 50% female) were healthy individuals between 20 and 60 years old with no restriction for race. All volunteers signed an informed consent form. People professionally involved in adenovirus research were

15   excluded.
Approximately 60 ml blood was drawn in dry tubes. Within two hours after sampling, the blood was centrifuged at 2500 rpm for 10 minutes. Approximately 30 ml serum was transferred to polypropylene tubes and stored frozen at -20°C until further

20   use.
Serum was thawed and heat-inactivated at 56°C for 10 minutes and then aliquotted to prevent repeated cycles of freeze/thawing. Part was used to make five steps of twofold dilutions in medium (DMEM, Gibco BRL) in a quantity enough

25   to fill out approximately 70 96-well plates. Aliquots of undiluted and diluted sera were pipetted in deep well plates (96-well format) and using a programmed platemate dispensed in 100 μl aliquots into 96-well plates. This way the plates were loaded with eight different sera in duplo (100 μl/well)

30   according to the scheme below:

S1/ 2	S1/ 4	S1/ 8	S1/ 16	S1/ 32	S5/ 2	S5/ 4	S5/ 8	S5/ 16	S5/ 32	–	–
S1/ 2	S1/ 4	S1/ 8	S1/ 16	S1/ 32	S5/ 2	S5/ 4	S5/ 8	S5/ 16	S5/ 32	–	–
S2/ 2	S2/ 4	S2/ 8	S2/ 16	S2/ 32	S6/ 2	S6/ 4	S6/ 8	S6/ 16	S6/ 32	–	–
S2/ 2	S2/ 4	S2/ 8	S2/ 16	S2/ 32	S6/ 2	S6/ 4	S6/ 8	S6/ 16	S6/ 32	–	–
S3/ 2	S3/ 4	S3/ 8	S3/ 16	S3/ 32	S7/ 2	S7/ 4	S7/ 8	S7/ 16	S7/ 32	–	–
S3/ 2	S3/ 4	S3/ 8	S3/ 16	S3/ 32	S7/ 2	S7/ 4	S7/ 8	S7/ 16	S7/ 32	–	–
S4/ 2	S4/ 4	S3/ 8	S3/ 16	S3/ 32	S8/ 2	S8/ 4	S8/ 8	S8/ 16	S8/ 32	–	–
S4/ 2	S4/ 4	S3/ 8	S3/ 16	S3/ 32	S8/ 2	S8/ 4	S8/ 8	S8/ 16	S8/ 32	–	–

Where S1/2 to S8/2 in columns 1 and 6 represent 1x diluted
sera and Sx/4, Sx/8, Sx/16 and Sx/32 the twofold serial

5　dilutions. The last plates also contained four wells filled
with 100 ul foetal calf serum as a negative control.
Plates were kept at -20°C until further use.

Preparation of human adenovirus stocks

10　Prototypes of all known human adenoviruses were inoculated
on T25 flasks seeded with PER.C6 cells (ECACC deposit number
96022940) (Fallaux et al., 1998) and harvested upon full
CPE. After freeze/thawing 1-2 ml of the crude lysates was
used to inoculate a T80 flask with PER.C6 cells (ECACC

15　deposit number 96022940) and virus was harvested at full
CPE. The timeframe between inoculation and occurrence of CPE
as well as the amount of virus needed to re-infect a new
culture, differed between serotypes. Adenovirus stocks were
prepared by freeze/thawing and used to inoculate 3-4 T175

20　cm^2 three-layer flasks with PER.C6 cells (ECACC deposit
number 96022940). Upon occurrence of CPE, cells were
harvested by tapping the flask, pelleted and virus was
isolated and purified by a two step CsCl gradient as
follows. Cell pellets were dissolved in 50 ml 10 mM NaPO$_4$

25　buffer (pH 7.2) and frozen at -20°C. After thawing at 37°C,

5.6 ml sodium deoxycholate (5% w/v) was added. The solution
was mixed gently and incubated for 5-15 minutes at 37°C to
completely lyse the cells. After homogenizing the solution,
1875 μl 1M MgCl$_2$ was added. After the addition of 375 μl

5   DNAse (10 mg/ml) the solution was incubated for 30 minutes
at 37°C. Cell debri was removed by centrifugation at 1880xg
for 30 minutes at RT without brake. The supernatant was
subsequently purified from proteins by extraction with freon
(3x). The cleared supernatant was loaded on a 1M Tris/HCl

10 buffered cesiumchloride blockgradient (range: 1.2/1.4 gr/ml)
and centrifugated at 21000 rpm for 2.5 hours at 10°C. The
virus band is isolated after which a second purification
using a 1M Tris/HCl buffered continues gradient of 1.33
gr/ml of cesiumchloride was performed. The virus was then

15 centrifuged for 17 hours at 55000 rpm at 10°C. The virus
band is isolated and sucrose (50 % w/v) is added to a final
concentration of 1%. Excess cesiumchloride is removed by
dialysis (three times 1 hr at RT) in dialysis slides (Slide-
a-lizer, cut off 10000 kDa, Pierce, USA) against 1.5 ltr PBS

20 supplemented with CaCl$_2$ (0.9 mM), MgCl$_2$ (0.5mM) and an
increasing concentration of sucrose (1, 2, 5%). After
dialysis, the virus is removed from the slide-a-lizer after
which it is aliquoted in portions of 25 and 100 μl upon
which the virus is stored at -85°C.

25 To determine the number of virus particles per milliliter,
50 μl of the virus batch is run on a high-pressure liquid
chromatograph (HPLC) as described by Shabram et al (1997).
Viruses were eluted using an NaCl gradient ranging from 0 to
600 mM. As depicted in table I, the NaCl concentration by

30 which the viruses were eluted differed significantly among
serotypes.
        Most human adenoviruses replicated well on PER.C6
cells ((ECACC deposit number 96022940) with a few
exceptions. Adenovirus type 8 and 40 were grown on 911-E4

35 cells (He et al., 1998). Purified stocks contained between
5x10^{10} and 5x10^{12} virus particles/ml (VP/ml; see table I).

Titration of purified human adenovirus stocks

Adenoviruses were titrated on PER.C6 cells (ECACC deposit
number 96022940) to determine the amount of virus necessary
to obtain full CPE in five days, the length of the
neutralisation assay. Hereto, 100 μl medium was dispensed
into each well of 96-well plates. 25 μl of adenovirus
stocks prediluted 10^4, 10^5, 10^6 or 10^7 times were added to
column 2 of a 96-well plate and mixed by pipetting up and
down 10 times. Then 25 μl was brought from column 2 to
column 3 and again mixed. This was repeated until column 11
after which 25 μl from column 11 was discarded. This way
serial dilutions in steps of 5 were obtained starting off
from a prediluted stock. Then 3×10^4 PER.C6 cells (ECACC
deposit number 96022940) were added in a 100 μl volume and
the plates were incubated at 37 °C, 5% CO_2 for five or six
days. CPE was monitored microscopically. The method of Reed
and Muensch was used to calculate the cell culture
inhibiting dose 50% (CCID50).

In parallel identical plates were set up that were
analysed using the MTT assay (Promega). In this assay living
cells are quantified by colorimetric staining. Hereto, 20 μl
MTT (7.5 mgr/ml in PBS) was added to the wells and incubated
at 37 °C, 5% CO_2 for two hours. The supernatant was removed
and 100 μl of a 20:1 isopropanol/triton-X100 solution was
added to the wells. The plates were put on a 96-wells shaker
for 3-5 minutes to solubilise precipitated staining.
Absorbance was measured at 540 nm and at 690 nm
(background). By this assay wells with proceeding CPE or
full CPE can be distinguished.

Neutralisation assay

96-well plates with diluted human serum samples were thawed
at 37 °C, 5% CO_2. Adenovirus stocks diluted to 200 CCID50
per 50 μl were prepared and 50 μl aliquots were added to
columns 1-11 of the plates with serum. Plates were incubated

21

for 1 hour at 37°C, 5% CO_2. Then 50 μl PER.C6 cells (ECACC deposit number 96022940) at 6×10^5/ml were dispensed in all wells and incubated for 1 day at 37 °C, 5% CO_2. Supernatant was removed using fresh pipet tips for each row and 200 μl

5   fresh medium was added to all wells to avoid toxic effects of the serum. Plates were incubated for another 4 days at 37 °C, 5% CO_2. In addition, parallel control plates were set up in duplo with diluted positive control sera generated in rabbits and specific for each serotype to be tested in rows

10   A and B and with negative control serum (FCS) in rows C and D. Also, in each of the rows E-H a titration was performed as described above with steps of five times dilutions starting with 200 CCID50 of each virus to be tested. On day 5 one of the control plates was analysed microscopically and

15   with the MTT assay. The experimental titer was calculated from the control titration plate observed microscopically. If CPE was found to be complete, i.e. the first dilution in the control titration experiment analysed by MTT shows clear cell death, all assay plates were processed. If not, the

20   assay was allowed to proceed for one or more days until full CPE was apparent after which all plates were processed. In most cases the assay was terminated at day 5. For Ad1, 5, 33, 39, 42 and 43 the assay was left for six days and for Ad2 for eight days.

25   A serum sample is regarded to be non-neutralising when at the highest serum concentration a maximum protection is seen of 40% compared to the controls without serum.
The results of the analysis of 44 prototype adenoviruses against serum from 100 healthy volunteers is shown in figure

30   1. As expected the percentage of serum samples that contained neutralising antibodies to Ad2 and Ad5 was very high. This was also true for most of the lower numbered adenoviruses. Surprisingly, none of the serum samples contained neutralising antibodies to adenovirus serotype 35.

35   Also, the number of individuals with neutralising antibody titers to the serotypes 26, 34 and 48 was very low.

Therefor, recombinant E1-deleted adenoviruses based on Ad35
or one of the other above mentioned serotypes have an
important advantage compared to recombinant vectors based on
Ad5 with respect to clearence of the viruses by neutralising

5   antibodies.
Also, Ad5-based vectors that have (parts of) the capsid
proteins involved in immunogenic response of the host
replaced by the corresponding (parts of) the capsid proteins
of Ad35 or one of the other serotypes will be less, or even

10   not, neutralised by the vast majority of human sera.
As can be seen in table I the VP/CCID50 ratio calculated
from the virus particles per ml and the CCID50 obtained for
each virus in the experiments was highly variable and ranged
from 0.4 to 5 log. This is probably caused by different

15   infection efficiencies of PER.C6 cells (ECACC deposit number
96022940) and by differences in replication efficiency of
the viruses. Furthermore, differences in batch qualities may
play a role. A high VP/CCID50 ratio means that more virus
was put in the wells to obtain CPE in 5 days. As a

20   consequence the outcome of the neutralisation study might be
biased since more (inactive) virus particles could shield
the antibodies. To check whether this phenomenon had taken
place, the VP/CCID50 ratio was plotted against the
percentage of serum samples found positive in the assay

25   (Figure 2). The graph clearly shows that there is no
negative correlation between the amount of viruses in the
assay and neutralisation in serum.

Example 2

<u>Generation of Ad5 plasmid vectors for the production of</u>

5   <u>recombinant viruses and easy manipulation of adenoviral</u>
<u>genes</u>
pBr/Ad.Bam-rITR (ECACC deposit P97082122)
In order to facilitate blunt end cloning of the ITR
sequences, wild-type human adenovirus type 5 (Ad5) DNA was

10   treated with Klenow enzyme in the presence of excess dNTPs.
After inactivation of the Klenow enzyme and purification by
phenol/chloroform extraction followed by ethanol
precipitation, the DNA was digested with BamHI. This DNA
preparation was used without further purification in a

15   ligation reaction with pBr322 derived vector DNA prepared as
follows: pBr322 DNA was digested with EcoRV and BamHI,
dephosphorylated by treatment with TSAP enzyme (Life
Technologies) and purified on LMP agarose gel (SeaPlaque
GTG). After transformation into competent *E.coli* DH5α (Life

20   Techn.) and analysis of ampiciline resistant colonies, one
clone was selected that showed a digestion pattern as
expected for an insert extending from the BamHI site in Ad5
to the right ITR.
Sequence analysis of the cloning border at the right ITR

25   revealed that the most 3' G residue of the ITR was missing,
the remainder of the ITR was found to be correct. Said
missing G residue is complemented by the other ITR during
replication.

30   *pBr/Ad.Sal-rITR (ECACC deposit P97082119)*
pBr/Ad.Bam-rITR was digested with BamHI and SalI. The vector
fragment including the adenovirus insert was isolated in LMP
agarose (SeaPlaque GTG) and ligated to a 4.8 kb SalI-BamHI
fragment obtained from wt Ad5 DNA and purified with the

35   Geneclean II kit (Bio 101, Inc.). One clone was chosen and
the integrity of the Ad5 sequences was determined by

restriction enzyme analysis. Clone pBr/Ad.Sal-rITR contains
adeno type 5 sequences from the SalI site at bp 16746 up to
and including the rITR (missing the most 3' G residue).

5   *pBr/Ad.Cla-Bam (ECACC deposit P97082117)*
wt Adeno type 5 DNA was digested with ClaI and BamHI, and
the 20.6 kb fragment was isolated from gel by electro-
elution. pBr322 was digested with the same enzymes and
purified from agarose gel by Geneclean. Both fragments were
10   ligated and transformed into competent DH5α. The resulting
clone pBr/Ad.Cla-Bam was analysed by restriction enzyme
digestion and shown to contain an insert with adenovirus
sequences from bp 919 to 21566.

15   *pBr/Ad.AflII-Bam (ECACC deposit P97082114)*
Clone pBr/Ad.Cla-Bam was linearized with EcoRI (in pBr322)
and partially digested with AflII. After heat inactivation
of AflII for 20' at 65°C the fragment ends were filled in
with Klenow enzyme. The DNA was then ligated to a blunt
20   double stranded oligo linker containing a PacI site (5'-
AATTGTCTTAATTAACCGCTTAA-3'). This linker was made by
annealing the following two oligonucleotides: 5'-
AATTGTCTTAATTAACCGC-3' and 5'-AATTGCGGTTAATTAAGAC-3',
followed by blunting with Klenow enzyme. After precipitation
25   of the ligated DNA to change buffer, the ligations were
digested with an excess PacI enzyme to remove concatameres
of the oligo. The 22016 bp partial fragment containing Ad5
sequences from bp 3534 up to 21566 and the vector sequences,
was isolated in LMP agarose (SeaPlaque GTG), religated and
30   transformed into competent DH5α. One clone that was found to
contain the PacI site and that had retained the large adeno
fragment was selected and sequenced at the 5' end to verify
correct insertion of the PacI linker in the (lost) AflII
site.

35

pBr/Ad.Bam-rITRpac#2 (ECACC deposit P97082120) and

pBr/Ad.Bam-rITRpac#8 (ECACC deposit P97082121)

To allow insertion of a PacI site near the ITR of Ad5 in clone pBr/Ad.Bam-rITR about 190 nucleotides were removed

5   between the ClaI site in the pBr322 backbone and the start of the ITR sequences. This was done as follows: pBr/Ad.Bam-rITR was digested with ClaI and treated with nuclease Bal31 for varying lengths of time (2', 5', 10' and 15'). The extend of nucleotide removal was followed by separate

10   reactions on pBr322 DNA (also digested at the ClaI site), using identical buffers and conditions. Bal31 enzyme was inactivated by incubation at 75 °C for 10', the DNA was precipitated and resuspended in a smaller volume TE buffer. To ensure blunt ends, DNAs were further treated with T4 DNA

15   polymerase in the presence of excess dNTPs. After digestion of the (control) pBr322 DNA with SalI, satisfactory degradation (~150 bp) was observed in the samples treated for 10' or 15'. The 10' or 15' treated pBr/Ad.Bam-rITR samples were then ligated to the above described blunted

20   PacI linkers (See pBr/Ad.AflII-Bam). Ligations were purified by precipitation, digested with excess PacI and separated from the linkers on an LMP agarose gel. After religation, DNAs were transformed into competent DH5α and colonies analyzed. Ten clones were selected that showed a deletion of

25   approximately the desired length and these were further analyzed by T-track sequencing (T7 sequencing kit, Pharmacia Biotech). Two clones were found with the PacI linker inserted just downstream of the rITR. After digestion with PacI, clone #2 has 28 bp and clone #8 has 27 bp attached to

30   the ITR.

pWE/Ad.AflII-rITR (ECACC deposit P97082116)

Cosmid vector pWE15 (Clontech) was used to clone larger Ad5 inserts. First, a linker containing a unique PacI site was

35   inserted in the EcoRI sites of pWE15 creating pWE.pac. To this end, the double stranded PacI oligo as described for

pBr/Ad.AflII-BamHI was used but now with its EcoRI
protruding ends. The following fragments were then isolated
by electro-elution from agarose gel: pWE.pac digested with
PacI, pBr/AflII-Bam digested with PacI and BamHI and
5   pBr/Ad.Bam-rITR#2 digested with BamHI and PacI. These
fragments were ligated together and packaged using λ phage
packaging extracts (Stratagene) according to the
manufacturer's protocol. After infection into host bacteria,
colonies were grown on plates and analyzed for presence of
10   the complete insert. pWE/Ad.AflII-rITR contains all
adenovirus type 5 sequences from bp 3534 (AflII site) up to
and including the right ITR (missing the most 3' G residue).

pBr/Ad.lITR-Sal(9.4) (ECACC deposit P97082115)

15   Adeno 5 wt DNA was treated with Klenow enzyme in the
presence of excess dNTPs and subsequently digested with
SalI. Two of the resulting fragments, designated left ITR-
Sal(9.4) and Sal(16.7)-right ITR, respectively, were
isolated in LMP agarose (Seaplaque GTG). pBr322 DNA was
20   digested with EcoRV and SalI and treated with phosphatase
(Life Technologies). The vector fragment was isolated using
the Geneclean method (BIO 101, Inc.) and ligated to the Ad5
SalI fragments. Only the ligation with the 9.4 kb fragment
gave colonies with an insert. After analysis and sequencing
25   of the cloning border a clone was chosen that contained the
full ITR sequence and extended to the SalI site at bp 9462.

pBr/Ad.lITR-Sal(16.7) (ECACC deposit P97082118)
pBr/Ad.lITR-Sal(9.4) is digested with SalI and
30   dephosphorylated (TSAP, Life Technologies). To extend this
clone upto the third SalI site in Ad5, pBr/Ad.Cla-Bam was
linearized with BamHI and partially digested with SalI. A
7.3 kb SalI fragment containing adenovirus sequences from
9462-16746 was isolated in LMP agarose gel and ligated to
35   the SalI-digested pBr/Ad.lITR-Sal(9.4) vector fragment.

pWE/Ad.AflII-EcoRI

pWE.pac was digested with ClaI and 5' protruding ends were
filled using Klenow enzyme. The DNA was then digested with
5   PacI and isolated from agarose gel. pWE/AflII-rITR was
digested with EcoRI and after treatment with Klenow enzyme
digested with PacI. The large 24 kb fragment containing the
adenoviral sequences was isolated from agarose gel and
ligated to the ClaI-digested and blunted pWE.pac vector
10 using the Ligation Express™ kit from Clontech. After
transformation of Ultracompetent XL10-Gold cells from
Stratagene, clones were identified that contained the
expected insert. pWE/AflII-EcoRI containes Ad5 sequences
from bp 3534-27336.
15

Generation of pWE/Ad.AflII-rITRsp
The 3' ITR in the vector pWE/Ad.AflII-rITR does not include
the terminal G-nucleotide. Furthermore, the PacI site is
located almost 30 bp from the right ITR. Both these
20 characteristics may decrease the efficiency of virus
generation due to inefficient initiation of replication at
the 3' ITR. Note that during virus generation the left ITR
in the adapter plasmid is intact and enables replication of
the virus DNA after homologous recombination.
25 To improve the efficiency of initiation of replication at
the 3' ITR, the pWE/Ad.AflII-rITR was modified as follows:
construct pBr/Ad.Bam-rITRpac#2 was first digested with PacI
and then partially digested with AvrII and the 17.8 kb
vector containing fragment was isolated and dephophorylated
30 using SAP enzyme (Boehringer Mannheim). This fragment lacks
the adenosequences from nucleotide 35464 to the 3'ITR. Using
DNA from pWE/Ad.AflII-rITR as template and the primers
ITR-EPH:
5'-CGG AAT TCT TAA TTA AGT TAA CAT CAT CAA TAA TAT ACC-3'
35 and
Ad101: 5'-TGA TTC ACA TCG GTC AGT GC-3'

28

a 630 bp PCR fragment was generated corresponding to the 3'
Ad5 sequences. This PCR fragment was subsequently cloned in
the vector pCR2.1 (Invitrogen) and clones containing the PCR
fragment were isolated and sequenced to check correct

5   amplification of the DNA. The PCR clone was then digested
with PacI and AvrII and the 0.5 kb adeno insert was ligated
to the PacI/ partial AvrII digested pBr/Ad.Bam-rITRpac#2
fragment generating pBr/Ad.Bam-rITRsp. Next this construct
was used to generate a cosmid clone (as described above)

10   that has an insert corresponding to the adenosequences 3534
to 35938. This clone was named pWE/AflII-rITRsp.

Generation of pWE/Ad.AflII-rITRΔE2A:

Deletion of the E2A coding sequences from pWE/Ad.AflII-rITR

15   (ECACC deposit P97082116) has been accomplished as follows.
The adenoviral sequences flanking the E2A coding region at
the left and the right site were amplified from the plasmid
pBr/Ad.Sal.rITR (ECACC deposit P97082119) in a PCR reaction
with the Expand PCR system (Boehringer) according to the

20   manufacturers protocol. The following primers were used:
Right flanking sequences (corresponding Ad5 nucleotides
24033 to 25180):

     ΔE2A.SnaBI: 5'-GGC GTA CGT AGC CCT GTC GAA AG-3'

     ΔE2A.DBP-start: 5'-CCA <u>ATG CAT</u> TCG AAG TAC TTC CTT

25   CTC CTA TAG GC-3'

The amplified DNA fragment was digested with SnaBI and NsiI
(NsiI site is generated in the primer ΔE2A.DBP-start,
underlined).
Left flanking sequences (corresponding Ad5 nucleotides 21557

30   to 22442):

     ΔE2A.DBP-stop: 5'-CCA <u>ATG CAT</u> ACG GCG CAG ACG G-3'

     ΔE2A.BamHI: 5'-GAG GTG GAT CCC ATG GAC GAG-3'

The amplified DNA was digested with BamHI and NsiI (NsiI
site is generated in the primer ΔE2A.DBP-stop, underlined).

35   Subsequently, the digested DNA fragments were ligated into

SnaBI/BamHI digested pBr/Ad.Sal-rITR. Sequencing confirmed
the exact replacement of the DBP coding region with a unique
NsiI site in plasmid pBr/Ad.Sal-rITRΔE2A. The unique NsiI
site can be used to introduce an expression cassette for a
5   gene to be transduced by the recombinant vector.
        The deletion of the E2A coding sequences was performed
such that the splice acceptor sites of the 100K encoding L4-
gene at position 24048 in the top strand was left intact. In
addition, the poly adenylation signals of the original E2A-
10   RNA and L3-RNAs at the left hand site of the E2A coding
sequences were left intact. This ensures proper expression
of the L3-genes and the gene encoding the 100K L4-protein
during the adenovirus life cycle.
        Next, the plasmid pWE/Ad.AflII-rITRΔE2A was generated. The
15   plasmid pBr/Ad.Sal-rITRΔE2A was digested with BamHI and
SpeI. The 3.9-Kb fragment in which the E2A coding region was
replaced by the unique NsiI site was isolated. The
pWE/Ad.AflII-rITR was digested with BamHI and SpeI. The 35
Kb DNA fragment, from which the BamHI/SpeI fragment
20   containing the E2A coding sequence was removed, was
isolated. The fragments were ligated and packaged using λ
phage-packaging extracts according to the manufacturer
protocol (Stratagene), yielding the plasmid pWE/Ad.AflII-
rITRΔE2A.
25   This cosmid clone can be used to generate adenoviral vectors
that are deleted for E2A by cotransfection of PacI digested
DNA together with digested adapter plasmids onto packaging
cells that express functional E2A gene product.

30   *Construction of adapter plasmids*
        The absence of sequence overlap between the recombinant
adenovirus and E1 sequences in the packaging cell line is
essential for safe, RCA-free generation and propagation of
new recombinant viruses. The adapter plasmid pMLPI.TK
35   (described in PCT/NL96/00244) is an example of an adapter
        plasmid designed for use according to the invention in

combination with the improved packaging cell lines of the
invention. This plasmid was used as the starting material to
make a new vector in which nucleic acid molecules comprising
specific promoter and gene sequences can be easily
5   exchanged.
First, a PCR fragment was generated from pZipΔMo+PyF101(N⁻)
template DNA (described in PCT/NL96/00195) with the
following primers: LTR-1: 5'-CTG TAC GTA CCA GTG CAC TGG CCT
AGG CAT GGA AAA ATA CAT AAC TG-3' and LTR-2: 5'-GCG GAT CCT
10 TCG AAC CAT GGT AAG CTT GGT ACC GCT AGC GTT AAC CGG GCG ACT
CAG TCA ATC G-3'. Pwo DNA polymerase (Boehringer Mannheim)
was used according to manufacturers protocol with the
following temperature cycles: once 5' at 95°C; 3' at 55°C;
and 1' at 72°C, and 30 cycles of 1' at 95°C, 1' at 60°C, 1'
15 at 72°C, followed by once 10' at 72°C. The PCR product was
then digested with BamHI and ligated into pMLP10 (Levrero et
al., 1991) vector digested with PvuII and BamHI, thereby
generating vector pLTR10. This vector contains adenoviral
sequences from bp 1 up to bp 454 followed by a promoter
20 consisting of a part of the Mo-MuLV LTR having its wild-type
enhancer sequences replaced by the enhancer from a mutant
polyoma virus (PyF101). The promoter fragment was designated
L420. Next, the coding region of the murine HSA gene was
inserted. pLTR10 was digested with BstBI followed by Klenow
25 treatment and digestion with NcoI. The HSA gene was obtained
by PCR amplification on pUC18-HSA (Kay et al., 1990) using
the following primers: HSA1, 5'-GCG CCA CCA TGG GCA GAG CGA
TGG TGG C-3' and HSA2, 5'-GTT AGA TCT AAG CTT GTC GAC ATC
GAT CTA CTA ACA GTA GAG ATG TAG AA-3'. The 269 bp amplified
30 fragment was subcloned in a shuttle vector using the NcoI
and BglII sites. Sequencing confirmed incorporation of the
correct coding sequence of the HSA gene, but with an extra
TAG insertion directly following the TAG stop codon. The
coding region of the HSA gene, including the TAG duplication
35 was then excised as a NcoI(sticky)-SalI(blunt) fragment and

cloned into the 3.5 kb NcoI(sticky)/BstBI(blunt) fragment from pLTR10, resulting in pLTR-HSA10.
Finally, pLTR-HSA10 was digested with EcoRI and BamHI after which the fragment containing the left ITR, packaging

5   signal, L420 promoter and HSA gene was inserted into vector pMLPI.TK digested with the same enzymes and thereby replacing the promoter and gene sequences. This resulted in the new adapter plasmid pAd/L420-HSA that contains convenient recognition sites for various restriction enzymes

10   around the promoter and gene sequences. SnaBI and AvrII can be combined with HpaI, NheI, KpnI, HindIII to exchange promoter sequences, while the latter sites can be combined with the ClaI or BamHI sites 3' from HSA coding region to replace genes in this construct.

15   Another adapter plasmid that was designed to allow easy exchange of nucleic acid molecules was made by replacing the promoter, gene and poly A sequences in pAd/L420-HSA with the CMV promoter, a multiple cloning site, an intron and a poly-A signal. For this purpose, pAd/L420-HSA was digested with

20   AvrII and BglII followed by treatment with Klenow to obtain blunt ends. The 5.1 kb fragment with pBr322 vector and adenoviral sequences was isolated and ligated to a blunt 1570 bp fragment from pcDNA1/amp (Invitrogen) obtained by digestion with HhaI and AvrII followed by treatment with T4

25   DNA polymerase. This adapter plasmid was named pAd5/CLIP. To enable removal of vector sequences from the left ITR in pAd5/Clip, this plasmid was partially digested with EcoRI and the linear fragment was isolated. An oligo of the sequence 5' TTAAGTCGAC-3' was annealed to itself resulting

30   in a linker with a SalI site and EcoRI overhang. The linker was ligated to the partially digested pAd5/Clip vector and clones were selected that had the linker inserted in the EcoRI site 23 bp upstream of the left adenovirus ITR in pAd5/Clip resulting in pAd5/Clipsal. Likewise, the EcoRI

35   site in pAd5/Clip has been changed to a PacI site by insertion of a linker of the sequence 5'-

AATTGTCTTAATTAACCGCAATT-3'. The pAd5/Clip vector was
partially digested with EcoRI, dephosphorylated and ligated
to the PacI linker with EcoRI overhang. The ligation mixture
was digested with PacI to remove concatamers, isolated from
5   agarose gel and religated. The resulting vector was named
pAd5/Clippac. These changes enable more flexibility to
liberate the left ITR from the plasmid vector sequences.
The vector pAd5/L420-HSA was also modified to create a SalI
or PacI site upstream of the left ITR. Hereto pAd5/L420-HSA
10   was digested with EcoRI and ligated to the above described
PacI linker. The ligation mixture was digested with PacI and
religated after isolation of the linear DNA from agarose gel
to remove concatamerised linkers. This resulted in adapter
plasmid pAd5/L420-HSApac. This construct was used to
15   generate pAd5/L420-HSAsal as follows: pAd5/L420-HSApac was
digested with ScaI and BsrGI and the vector fragment was
ligated to the 0.3 kb fragment isolated after digestion of
pAd5/Clipsal with the same enzymes.

20   *Generation of adapter plasmids pAdMire and pAdApt*
To create an adapter plasmid that only contains a polylinker
sequence and no promoter or polyA sequences, pAd5/L420-
HSApac was digested with AvrII and BglII. The vector
fragment was ligated to a linker oligonucleotide digested
25   with the same restriction enzymes. The linker was made by
annealing oligos of the following sequence:
PLL-1: 5'- GCC ATC CCT AGG AAG CTT GGT ACC GGT GAA TTC GCT
AGC GTT AAC GGA TCC TCT AGA CGA GAT CTG G-3' and
PLL-2: 5'- CCA GAT CTC GTC TAG AGG ATC CGT TAA CGC TAG CGA
30   ATT CAC CGG TAC CAA GCT TCC TAG GGA TGG C-3'.
The annealed linkers were digested with AvrII and BglII and
separated from small ends by column purification (Qiaquick
nucleotide removal kit) according to manufacterers
recommendations. The linker was then ligated to the
35   AvrII/BglII digested pAd5/L420-HSApac fragment. A clone,

named AdMire, was selected that had the linker incorporated and was sequenced to check the integrity of the insert. Adapter plasmid AdMire enables easy insertion of complete expression cassettes.

5   An adapter plasmid containing the human CMV promoter that mediates high expression levels in human cells was constructed as follows: pAd5/L420-HSApac was digested with AvrII and 5' protruding ends were filled in using Klenow enzyme. A second digestion with HindIII resulted in removal

10   of the L420 promoter sequences. The vector fragment was isolated and ligated to a PCR fragment containing the CMV promoter sequence. This PCR fragment was obtained after amplification of CMV sequences from pCMVLacI (Stratagene) with the following primers:

15   CMVplus: 5'-GATCGGTACCA<u>CTGCAG</u>TGGTCAATATTGGCCATTAGCC-3' and
     CMVminA: 5'-GATC<u>AAGCTT</u>CCAATGCACCGTTCCCGGC-3'.
     The PCR fragment was first digested with PstI (underlined in CMVplus) after which the 3'-protruding ends were removed by treatment with T4 DNA polymerase. Then the DNA was digested

20   with HindIII (underlined in CMVminA) and ligated into the above described pAd5/L420-HSApac vector fragment digested with AvrII and HindIII. The resulting plasmid was named pAd5/CMV-HSApac. This plasmid was then digested with HindIII and BamHI and the vector fragment was isolated and ligated

25   to the polylinker sequence obtained after digestion of AdMire with HindIII and BglII. The resulting plasmid was named pAdApt. Adapter plasmid pAdApt contains nucleotides -735 to +95 of the human CMV promoter (Boshart et al., 1985). A second version of this adapter plasmid containing a SalI

30   site in place of the PacI site upstream of the left ITR was made by inserting the 0.7 kb ScaI-BsrGI fragment from pAd5/Clipsal into pAdApt digested with ScaI and partially digested with BsrGI. This clone was named pAdApt.sal.

Generation of recombinant adenoviruses based on Ad5

RCA free recombinant adenoviruses can be generated very
efficiently using the above described adapter plasmids and
the pWe/Ad.AflII-rITR or pWE/Ad.AflII-rITrsp constructs.

5   Generally, the adapter plasmid containing the desired
transgene in the desired expression cassette is digested
with suitable enzymes to liberate the insert from vector
sequences at the 3' and/or at the 5' end. The adenoviral
complementation plasmids pWE/Ad.AflII-rITR or pWE/Ad.AflII-

10   rITRsp are digested with PacI to liberate the adeno
sequences from the vector plasmids. As a non-limiting
example the generation of AdApt-LacZ is described. Adapter
plasmid pAdApt-LacZ was generated as follows. The E.coli
LacZ gene was amplified from the plasmid pMLP.nlsLacZ (EP

15   95-202 213) by PCR with the primers 5'-
GGGGTGGCCAGGGTACCTCTAGGCTTTTGCAA-3' and 5'-
GGGGGGATCCATAAACAAGTTCAGAATCC-3'. The PCR reaction was
performed with Ex Taq (Takara) according to the suppliers
protocol at the following amplification program: 5 minutes

20   94°C, 1 cycle; 45 seconds 94°C and 30 seconds 60°C and 2
minutes 72°C, 5 cycles; 45 seconds 94°C and 30 seconds 65°C
and 2 minutes 72°C, 25 cycles; 10 minutes 72; 45 seconds
94°C and 30 seconds 60°C and 2 minutes 72°C, 5 cycles, I
cycle. The PCR product was subsequently digested with Kpn1

25   and BamHI and the digested DNA fragment was ligated into
KpnI/BamHI digested pcDNA3 (Invitrogen), giving rise to
pcDNA3.nlsLacZ. Construct pcDNA3.nlsLacZ was then digested
with KpnI and BamHI and the 3 kb LacZ fragment was isolated
from gel using the geneclean spin kit (Bio 101, Inc.).

30   pAdApt was also digested with KpnI and BamHI and the linear
vector fragment was isolated from gel as above. Both
isolated fragments were ligated and one clone containing the
LacZ insert was selected. Construct pAdApt-LacZ was digested
with SalI, purified by the geneclean spin kit and

35   subsequently digested with PacI. pWE/Ad.AflII-rITRsp was
digested with PacI. Both digestion mixtures were treated for

30' by 65 °C to inactivate the enzymes. Samples were put on
gel to estimate the concentration. 2.5×10^6 PER.C6 cells
(ECACC deposit number 96022940) were seeded in T25 flasks in
DMEM with 10% FCS and 10mM MgCl. The next day four microgram

5   of each plasmid was transfected into PER.C6 cells (ECACC
deposit number 96022940) using lipofectamine transfection
reagence (Life Technologies Inc.) according to instructions
of the manufacturer. The next day the medium was replaced by
fresh culture medium and cells were further cultured at 37°

10   C, 10% CO_2. Again 24 hrs. later cells were trypsinised,
seeded into T80 flasks and cultured at 37°C, 10% CO_2. Full
CPE was obtained 6 days after seeding in the T80 flask.Cells
were harvested in the medium and subjected to one
freeze/thaw cycle. The crude lysate obtained this way was

15   used to plaque purify the mixture of viruses. Ten plaques
were picked, expanded in a 24 well plate and tested for LacZ
expression following infection of A549 cells. Viruses from
all ten plaques expressed LacZ.

20   **Example 3**

          Generation of chimeric recombinant adenoviruses

          Generation of hexon chimeric Ad5-based adenoviruses
25   Neutralising antibodies in human serum are mainly directed
to the hexon protein and to a lesser extend to the penton
protein. Hexon proteins from different serotypes show highly
variable regions present in loops that are predicted to be
exposed at the outside of the virus (Athappilly et al.,

30   1994; J. Mol. Biol. 242, 430-455). Most type specific
epitopes have been mapped to these highly variable regions
(Toogood et al., 1989; J. Gen Virol. 70, 3203-3214). Thus
replacement of (part of) the hexon sequences with
corresponding sequences from a different serotype is an

35   effective strategy to circumvent (pre-existing) neutralising

antibodies to Ad5. Hexon coding sequences of adenovirus
serotype 5 are located between nucleotides 18841 and 21697.
To facilitate easy exchange of hexon coding sequences from
alternative adenovirus serotypes into the adenovirus
5   serotype 5 backbone, first a shuttle vector was generated.
This subclone, coded pBr/Ad.Eco-PmeI, was generated by first
digesting plasmid pBr322 with EcoRI and EcoRV and inserting
the 14 kb PmeI-EcoRI fragment from pWE/Ad.AflII-Eco. In this
shuttle vector a deletion was made of a 1430 bp SanDI
10   fragment by digestion with SanDI and religation to give
pBr/Ad.Eco-PmeI ΔSanDI. The removed fragment contains unique
SpeI and MunI sites. From pBr/Ad.Eco-PmeIΔSanDI the
adenovirus serotype 5 DNA encoding hexon was deleted.
Hereto, the hexon flanking sequences were PCR amplified and
15   linked together thereby generating unique restriction sites
replacing the hexon coding region. For these PCR reactions
four different oligonucleotides were required: Δhex1-Δnex4.
Δhex1: 5'- CCT GGT GCT GCC AAC AGC-3'
Δhex2: 5'- CCG **GAT CC**A CTA GTG GAA AGC GGG CGC GCG-3'
20   Δhex3: 5'- CCG **GAT CC**A ATT GAG AAG CAA GCA ACA TCA ACA AC-3'
Δhex4: 5'- GAG AAG GGC ATG GAG GCT G-3'
The amplified DNA product of ± 1100 bp obtained with
oligonucleotides Δhex1 and Δhex2 was digested with BamHI and
FseI. The amplified DNA product of ± 1600 bp obtained with
25   oligonucleotides Δhex3 and Δhex4 was digested with BamHI and
SbfI. These digested PCR fragments were subsequently
purified from agarose gel and in a tri-part ligation
reaction using T4 ligase enzyme linked to pBr/Ad.Eco-PmeI Δ
SanDI digested with FseI and SbfI. The resulting construct
30   was coded pBr/Ad.Eco-PmeΔHexon. This construct was sequenced
in part to confirm the correct nucleotide sequence and the
presence of unique restriction sites MunI and SpeI.
pBr/Ad.Eco-PmeΔHexon serves as a shuttle vector to introduce
heterologous hexon sequences amplified from virus DNA from
35   different serotypes using primers that introduce the unique

37

restriction sites MunI and SpeI at the 5' and 3' ends of the
hexon sequences respectively. To generate Ad5-based vectors
that contain hexon sequences from the serotypes to which
healthy individuals have no, or very low, titers of NAB the

5   hexon sequences of Ad35, Ad34, Ad26 and Ad48 were amplified
using the following primers:
Hex-up2: 5'-GACTAGTCAAGATGGCYACCCCHTCGATGATG-3' and
Hex-do2: 5'-GCTGGCCAATTGTTATGTKGTKGCGTTRCCGGC-3'.
These primers were designed using the sequences of published

10 hexon coding regions (for example hexon sequences of Ad2,
Ad3, Ad4, Ad5, Ad7, Ad16, Ad40 and Ad41 can be obtained at
Genbank). Degenerated nucleotides were incorporated at
positions that show variation between serotypes.

15 PCR products were digested with SpeI and MunI and cloned
into the pBr/Ad.Eco-PmeΔHexon construct digested with the
same enzymes.
The hexon modified sequences were subsequently introduced in
the construct pWE/Ad.AflII-rITR by exchange of the AscI

20 fragment generating pWE/Ad.AflII-rITRHexXX where XX stands
for the serotype used to amplify hexon sequences.   ·
The pWE/Ad.AflII-rITRHexXX constructs are then used to make
viruses in the same manner as described above for Ad5
recombinant viruses.

25
Generation of penton chimeric Ad5-based recombinant viruses
The adenovirus type 5 penton gene is located between
sequences 14156 and 15869. Penton base is the adenovirus
capsid protein that mediates internalisation of the virus

30 into the target cell. At least some serotypes (type C and B)
have been shown to achieve this by interaction of an RGD
sequence in penton with integrins on the cell surface.
However, type F adenoviruses do not have an RGD sequence and
for most viruses of the A and D group the penton sequence is

35 not known. Therefor, penton may be involved in target cell
specificity. Furthermore, as a capsid protein, the penton

protein is involved in the immunogenicity of the adenovirus
(Gahery-Segard et al., 1998). Therefor, replacement of Ad5
penton sequences with penton sequences from serotypes to
which no or low titers of NAB exist in addition to
5 replacement of the hexon sequences will prevent clearence of
the adenoviral vector more efficiently than replacement of
hexon alone. Replacement of penton sequences may also affect
infection specificity.
To be able to introduce heterologous penton sequences in Ad5
10 we made use of the plasmid-based system described above.
First a shuttle vector for penton sequences was made by
insertion of the 7.2 kb NheI-EcoRV fragment from construct
pWE/Ad.AflII-EcoRI into pBr322 digested with the same
enzymes. The resulting vector was named pBr/XN. From this
15 plasmid Ad5 penton sequences were deleted and replaced by
unique restriction sites that are then used to introduce new
penton sequences from other serotypes. Hereto, the left
flanking sequences of penton in pBr/XN were PCR amplified
using the following primers:
20 DP5-F: 5'- CTG TTG CTG CTG CTA ATA GC-3' and
DP5-R: 5'- CGC GGA TCC TGT ACA ACT AAG GGG AAT ACA AG-3'
DP5-R has an BamHI site (underlined) for ligation to the
right flanking sequence and also introduces a unique BsrGI
site (bold face) at the 5'-end of the former Ad5 penton
25 region.
The right flanking sequence was amplified using:
DP3-F: 5'-CGC GGA TCC CTT AAG GCA AGC ATG TCC ATC CTT-3' and
DP3-3R: 5'- AAA ACA CGT TTT ACG CGT CGA CCT TTC-3'
DP3-F has an BamHI site (underlined) for ligation to the
30 left flanking sequence and also introduces a unique AflII
site (bold face) at the 3'-end of the former Ad5 penton
region.
The two resulting PCR fragments were digested with BamHI and
ligated together. Then this ligation mixture was digested
35 with AvrII and BglII. pBr/XN was also digested with AvrII

39

and BglII and the vector fragment was ligated to the
digested ligated PCR fragments. The resulting clone was
named pBr/Ad.Δpenton. Penton coding sequences from Ad35,
Ad34, Ad26 and Ad48 were PCR amplified such that the 5' and
5   3' ends contained the BsrGI and AflII sites respectively.
Hereto, the following primers were used:
For Ad34 and Ad35:
P3-for: 5'-GCT CGA TGT ACA ATG AGG AGA CGA GCC GTG CTA-3'
P3-rev: 5'-GCT CGA CTT AAG TTA GAA AGT GCG GCT TGA AAG-3'
10   For Ad26 and Ad48:
P17F: 5'-GCT CGA TGT ACA ATG AGG CGT GCG GTG GTG TCT TC-3'
P17R: 5'-GCT CGA CTT AAG TTA GAA GGT GCG ACT GGA AAG C-3'

Amplified pcr products were digested with BfrI and BsrGI and
15   cloned into pBr/Ad.Δpenton digested with the same enzymes.
Introduction of these heterologous penton sequences in
pBr/Ad.Δpenton generated constructs named pBr/Ad.pentonXX
where XX represents the number of the serotype corresponding
to the serotype used to amplify the inserted penton
20   sequences. Subsequently the new penton sequences were
introduced in the a pWE/Ad.AflII-rITR vector having a
modified hexon. For example penton sequences from Ad35 were
introduced in the construct pWE/Ad.AflII-rITRHex35 by .
exchange of the common FseI fragment. Other combinations of
25   penton and hexon sequences were also made. Viruses with
modified hexon and penton sequences were made as described
above using cotransfection with an adapter plasmid on PER.C6
cells (ECACC deposit number 96022940). In addition, penton
sequences were introduced in the pWE/Ad.AflII-rITR
30   construct. The latter constructs contain only a modified
penton and viruses generated from these constructs will be
used to study the contribution of penton sequences to the
neutralisation of adenoviruses and also for analysis of
possible changes in infection efficiency and specificity.
35

Generation of fiber chimeric Ad5-based viruses

Adenovirus infection is mediated by two capsid proteins
fiber and penton. Binding of the virus to the cells is
achieved by interaction of the protruding fiber protein with
5   a receptor on the cell surface. Internalisation then takes
place after interaction of the penton protein with integrins
on the cell surface. At least some adenovirus from subgroup
C and B have been shown to use a different receptor for cell
binding and therefor have different infection efficiencies
10   on different cell types. Thus it is possible to change the
infection spectrum of adenoviruses by changing the fiber in
the capsid. The fiber coding sequence of adenovirus serotype
5 is located between nucleotides 31042 and 32787. To remove
the adenovirus serotype 5 DNA encoding fiber we started with
15   construct pBr/Ad.Bam-rITR. First a NdeI site was removed
from this construct. For this purpose, pBr322 plasmid DNA
was digested with NdeI after which protruding ends were
filled using Klenow enzym. This pBr322 plasmid was then re-
ligated, digested with NdeI and transformed into *E.coli* DH5α
20   . The obtained pBr/ΔNdeI plasmid was digested with ScaI and
SalI and the resulting 3198 bp vector fragment was ligated
to the 15349 bp ScaI-SalI fragment derived from
pBr/Ad.BamrITR, resulting in plasmid pBr/Ad.Bam-rITRΔNdeI
which hence contained a unique NdeI site. Next a PCR was
25   performed with oligonucleotides
NY-up:
5'- CGA **CAT ATG** TAG <u>ATG CAT</u> TAG TTT GTG TTA TGT TTC AAC GTG-3'
and
NY-down:
30   5'-GGA GAC CAC TGC CAT GTT-3'
During amplification, both a NdeI (bold face) and a NsiI
restriction site (underlined) were introduced to facilitate
cloning of the amplified fiber DNAs. Amplification consisted
of 25 cycles of each 45 sec. at 94°C, 1 min. at 60°C, and 45
35   sec. at 72°C. The PCR reaction contained 25 pmol of
oligonucleotides NY-up or NY-down, 2mM dNTP, PCR buffer with

1.5 mM MgCl$_2$, and 1 unit of Elongase heat stable polymerase
(Gibco, The Netherlands). One-tenth of the PCR product was
run on an agarose gel which demonstrated that the expected
DNA fragment of ± 2200 bp was amplified. This PCR fragment

5   was subsequently purified using Geneclean kit system (Bio101
Inc.). Then, both the construct pBr/Ad.Bam-rITRΔNdeI as well
as the PCR product were digested with restriction enzymes
NdeI and SbfI. The PCR fragment was subsequently cloned
using T4 ligase enzyme into the NdeI and SbfI digested

10   pBr/Ad.Bam-rITRΔNdeI, generating pBr/Ad.BamRΔFib.
This plasmid allows insertion of any PCR amplified fiber
sequence through the unique NdeI and NsiI sites that are
inserted in place of the removed fiber sequence. Viruses can
be generated by a double homologous recombination in

15   packaging cells described in patent No. PCT/NL96/00244 using
an adapter plasmid, construct pBr/Ad.AflII-EcoRI digested
with PacI and EcoRI and a pBr/Ad.BamRΔFib construct in which
heterologous fiber sequences have been inserted. To increase
the efficiency of virus generation, the construct

20   pBr/Ad.BamRΔFib was modified to generate a PacI site
flanking the right ITR. Hereto, pBr/Ad.BamRΔFib was digested
with AvrII and the 5 kb adenofragment was isolated and
introduced into the vector pBr/Ad.Bam-rITR.pac#8 described
above replacing the corresponding AvrII fragment. The

25   resulting construct was named pBr/Ad.BamRΔFib.pac.
Once a heterologous fiber sequence is introduced in
pBr/Ad.BamRΔFib.pac, the fiber modified right hand
adenovirus clone is introduced into a large cosmid clone as
described above for pWE/Ad.AflII-rITR. Such a large cosmid

30   clone allows generation of adenovirus by only one homologous
recombination. Ad5-based viruses with modified fibers have
been made and described (nos. 98204482.8 and 99200624.7). In
addition, hexon and penton sequences from serotypes from
this invention are combined with the desired fiber sequences

35   to generate viruses which infect the target cell of choice

very efficiently. For example smooth muscle cells,
endothelial cells or synoviocytes all from human origin are
very well infected with Ad5 based viruses with a fiber from
subgroup B viruses especially adenovirus type 16.

5   The above described examples in which specific sequences can
be deleted from the Ad5 backbone in the plasmids and
replaced by corresponding sequences from other serotypes
clearly show the flexibility of the system. It is evident
that by the methods described above any combination of

10   capsid gene from different serotypes can be made. Thus,
chimeric recombinant Ad5-based adenoviruses are designed
with desired hexon and penton sequences making the virus
less sensitive for neutralisation and with desired fiber
sequences allowing efficient infection in specific target

15   tissues.

Example 4

Construction of a plasmid-based system to generate Ad35
20   ## recombinant viruses

Partial restriction maps of Ad35 have been published
previously (Valderrama-Leon et al., 1985; Kang et al., 1989;
Li et al. 1991). An example of a functional plasmid-based

25   system to generate recombinant adenoviruses based on Ad35
consists of the following elements:

1. An adapter plasmid comprising a left ITR and packaging
   sequences derived from Ad35 and at least one restriction
   site for insertion of an heterologous expression cassette

30   and lacking E1 sequences. Furthermore, the adapter
   plasmid contains Ad35 sequences 3' from the E1B coding
   region including the pIX promoter and coding sequences
   sufficient to mediate homologous recombination of the
   adapter plasmid with a second nucleotide.

35   2. A second nucleotide comprising sequences homologous to
   the adapter plasmid and Ad35 sequences necessary for the

replication and packaging of the recombinant virus, that
is early, intermediate and late genes that are not
present in the packaging cell.

3. A packaging cell providing at least functional E1
5   proteins capable of complementing the E1 function of
         Ad35.

Ad35 DNA was isolated from a purified virus batch as
follows. To 100 μl of virus stock (Ad35: $3.26x10^{12}$ VP/ml)
10   10μl 10x DNAse buffer (130 mM Tris-HCl pH7.5; 1,2 M CaCl$_2$;
50mM MgCl$_2$) was added. After addition of 10 μl 10mgr/ml
DNAse I (Roche Diagnostics) the mixture was incubated for 1
hr. at 37°C. Following addition of 2.5μl 0.5M EDTA, 3.2μl
20% SDS and 1.5μl ProteinaseK (Roche Diagnostics; 20mgr/ml)
15   samples were incubated at 50°C for 1 hr. Next, the viral DNA
was isolated using the Geneclean spin kit (Bio101 Inc.)
according to the manufacters instructions. DNA was eluted
from the spin column with 25 μl sterile MilliQ water.
In the following sizes of DNA fragments and fragment
20   numbering will be used according to Kang et al. (1989). Ad35
DNA was digested with EcoRI and the three fragments
(approximately 22.3 (A), 7.3 (B) and 6 kb (C)) were isolated
from gel using the Geneclean kit (Bio101, Inc.). pBr322 was
digested with EcoRI or with EcoRI and EcoRV and digested
25   fragments were isolated from gel and dephosphorylated with
Tsap enzyme (Gibco BRL). Next, the 6 kb Ad35 C fragment was
ligated to the pBr322xEcoRI fragment and the ITR-containing
Ad35 fragment (EcoRI-B) was ligated to the
pBr322xEcoRI/EcoRV fragment. Ligations were incubated at
30   16°C overnight and transformed into DH5α competent bacteria
(Life Techn.). Minipreps of obtained colonies were analysed
for correct insertion of the Ad35 fragments by restriction
analysis. Both the 6 kb and the 7.3 kb Ad35 fragment were
found to be correctly inserted in pBr322. The 6kb fragment
35   was isolated in both orientations pBr/Ad35-Eco6.0⁺ and
pBr/Ad35-Eco6.0⁻ whereby the + stands for 5' to 3'

orientation relative to pBr322. The clone with the 7.3 kb
Ad35 B insert, named pBr/Ad35-Eco7.3 was partially sequenced
to check correct ligation of the 3' ITR. It was found that
the ITR had the sequence 5'- CATCATCAAT...-3' in the lower

5   strand. Then pBr/Ad35-Eco7.3 was extended to the 5' end by
insertion of the 6kb Ad35 fragment. Hereto, pBr/Ad35-Eco7.3
was digested with EcoRI and dephosphorylated. The fragment
was isolated from gel and ligated to the 6kb Ad35 EcoRI
fragment. After transformation clones were tested for

10   correct orientation of the insert and one clone was
selected, named pBr/Ad35-Eco13.3.
This clone is then extended with the ˜5.4 kb SalI D fragment
obtained after digestion of wt Ad35 with SalI. Hereto, the
SalI site in the pBr322 backbone is removed by partial

15   digestion of pBr/Ad35-Eco13.3 with SalI, filling in of the
sticky ends by Klenow treatment and religation. One clone is
selected that contains a single SalI site in the adenoviral
insert. This clone, named pBrΔsal /Ad35-Eco13.3 is then
linearised with AatII which is present in the pBr322

20   backbone and ligated to a SalI linker with AatII
complementary ends. The DNA is then digested with excess
SalI and the linear fragment is isolated and ligated to the
5.4 kb SalI-D fragment from Ad35. One clone is selected that
contains the SalI fragment inserted in the correct

25   orientation in pBr/Ad35-Eco13.3. The resulting clone,
pBr/Ad35.Sal2-rITR contains the 3' ˜17 kb of Ad35 including
the right ITR. To enable liberation of the right ITR from
the vector sequences at the time of virus generation, a NotI
site flanking the right ITR is introduced by PCR.

30   The Ad35 EcoRI-A fragment of 22.3 kb was also cloned in
pBr322xEcoRI/EcoRV. One clone, named pBr/Ad35-EcoA3', was
selected that apparently had a deletion of approximately 7kb
of the 5' end. It did contain the SalI site at 9.4 kb in
Ad35 wt DNA and approximately 1.5 kb of sequences upstream.

35   Using this SalI site and the unique NdeI site in the pBr322
backbone this clone is extended to the 5' end by insertion

of an approximately 5 kb Ad35 fragment 5' from the first
SalI in Ad35 in such a way that a NotI restriction site is
created at the 5' end of the Ad35 by insertion of a linker.
This clone, named pBr/Ad35.pIX-EcoA does not contain the
5   left end sequences (ITR, packaging sequences and E1) and at
the 3' end it has approximately 3.5 kb overlap with clone
pBr/Ad35.Sal2-rITR.
To create an adapter plasmid, Ad35 was digested with SalI
and the left end B fragment of ~9.4 kb was isolated. pBr322
10 was digested with EcoRV and SalI, isolated from gel and
dephosphorylated with Tsap enzyme. Both fragments are
ligated and clones with correct insertion and correct
sequence of the left ITR are selected. To enable liberation
of the left ITR from the vector sequences at the time of
15 virus generation, a NotI site flanking the left ITR is
introduced by PCR. From this clone the E1 sequences are
deleted and replaced by a polylinker sequence using PCR. The
polylinker sequence is used to introduce an expression
cassette for a gene of choice.
20 Recombinant Ad35 clones are generated by transfection of
PER.C6 cells with the adapter plasmid, pBr/Ad35.pIX-EcoA and
pBr/Ad35.Sal2-rITR as shown in figure 3. Homologous
recombination gives rise to recombinant viruses.

25   **Example 5**
The prevalence of neutralizing activity (NA) to Ad35 is low
in human sera from different geographic locations .

In example 1 we have described the analysis of
neutralizing activity (NA) in human sera from one location
30 in Belgium. Strikingly, of a panel of 44 adenovirus
serotypes tested, one serotype, Ad35, was not neutralized in
any of the 100 sera assayed. In addition, a few serotypes,
Ad26, Ad34 and Ad48 were found to be neutralized in 8%, or
less, of the sera tested. This analysis was further extended
35 to other serotypes of adenovirus not previously tested and,

46

using a selection of serotypes from the first screen, was
also extended to sera from different geographic locations.
        Hereto, adenoviruses were propagated, purified and
tested for neutralization in the CPE-inhibition assay as
5 described in example 1. Using the sera from the same batch
as in example 1, adenovirus serotypes 7B, 11, 14, 18 and
44/1876 were tested for neutralization. These viruses were
found to be neutralized in respectively 59, 13, 30, 98 and
54 % of the sera. Thus, of this series Ad11 is neutralized
10 with a relatively low frequency.
Since it is known that the frequency of isolation of
adenovirus serotypes from human tissue as well as the
prevalence of NA to adenovirus serotypes may differ on
different geographic locations, we further tested a
15 selection of the adenovirus serotypes against sera from
different places. Human sera were obtained from two
additional places in Europe (Bristol, UK and Leiden, The
Netherlands) and from two places in the United States
(Stanford, CA and Great Neck, NY). Adenoviruses that were
20 found to be neutralized in 20% or less of the sera in the
first screen, as well as Ad2, Ad5, Ad27, Ad30, Ad38, Ad43,
were tested for neutralization in sera from the UK. The
results of these experiments are presented in Figure 4.
Adenovirus serotypes 2 and 5 were again neutralized in a
25 high percentage of human sera. Furthermore, some of the
serotypes that were neutralized in a low percentage of sera
in the first screen are neutralized in a higher percentage
of sera from the UK, e.g. Ad26 (7% vs. 30%), Ad28 (13% vs.
50%), Ad34 (5% vs. 27%) and Ad48 (8% vs. 32%). Neutralizing
30 activity against Ad11 and Ad49 that were found in a
relatively low percentage of sera in the first screen, are
found in an even lower percentage of sera in this second
screen (13% vs. 5% and 20% vs. 11% respectively). Serotype
Ad35 that was not neutralized in any of the sera in the
35 first screen, was now found to be neutralized in a low
percentage (8%) of sera from the UK. The prevalence of NA in

human sera from the UK is the lowest to serotypes Ad11 and
Ad35.

5   For further analysis, sera were obtained from two
locations in the US (Stanford, CA and Great Neck, NY) and
from the Netherlands (Leiden). Figure 5 presents an overview
of data obtained with these sera and the previous data. Not
all viruses were tested in all sera, except for Ad5, Ad11
and Ad35. The overall conclusion from this comprehensive
screen of human sera is that the prevalence of neutralizing
10   activity to Ad35 is the lowest of all serotypes throughout
the western countries: on average 7% of the human sera
contain neutralizing activity (5 different locations).
Another B-group adenovirus, Ad11 is also neutralized in a
low percentage of human sera (average 11% in sera from 5
15   different locations). Adenovirus type 5 is neutralized in
56% of the human sera obtained from 5 different locations.
Although not tested in all sera, D-group serotype 49 is also
neutralized with relatively low frequency in samples from
Europe and from one location of the US (average 14%).
20   In the above described neutralization experiments a
serum is judged non-neutralizing when in the well with the
highest serum concentration the maximum protection of CPE is
40% compared to the controls without serum. The protection
is calculated as follows:

25

$$\% \text{ protection} = \frac{\text{OD corresponding well} - \text{OD virus control}}{\text{OD non-infected control} - \text{OD virus control}} \times 100 \%$$

30   As described in example 1, the serum is plated in five
different dilutions ranging from 4x to 64x diluted.
Therefore, it is possible to distinguish between low titers
(i.e. neutralization only in the highest serum
concentrations) and high titers of NA (i.e. also
35   neutralization in wells with the lowest serum
concentration). Of the human sera used in our screen that

were found to contain neutralizing activity to Ad5, 70%
turned out to have high titers whereas of the sera that
contained NA to Ad35, only 15% had high titers. Of the sera
that were positive for NA to Ad11 only 8% had high titers.

5   For Ad49 this was 5%. Therefore, not only is the frequency
of NA to Ad35, Ad11 and Ad49 much lower as compared to Ad5,
but of the sera that do contain NA to these viruses, the
vast majority has low titers. Adenoviral vectors based on
Ad11,Ad35 or Ad49 have therefore a clear advantage over Ad5

10   based vectors when used as gene therapy vehicles or
vaccination vectors *in vivo* or in any application where
infection efficiency is hampered by neutralizing activity.

    In the following examples the construction of a vector
system for the generation of safe, RCA-free Ad35-based

15   vectors is described.

                          Example 6
        Sequence of the human adenovirus type 35
    Ad35 viruses were propagated on PER.C6 cells and DNA

20   was isolated as described in example 4. The total sequence
was generated by Qiagen Sequence Services (Qiagen GmbH,
Germany). Total viral DNA was sheared by sonification and
the ends of the DNA were made blunt by T4 DNA polymerase.
Sheared blunt fragments were size fractionated on agarose

25   gels and gel slices corresponding to DNA fragments of 1.8 to
2.2 kb were obtained. DNA was purified from the gel slices
by the QIAquick gel extraction protocol and subcloned into a
shotgun library of pUC19 plasmid cloning vectors. An array
of clones in 96-wells plates covering the target DNA 8 (+/-

30   2) times was used to generate the total sequence. Sequencing
was performed on Perkin-Elmer 9700 thermo cyclers using
BigDyeTerminator chemistry and AmpliTaq FS DNA polymerase
followed by purification of sequencing reactions using
QIAGEN DyeEx 96 technology. Sequencing reaction products

35   were then subjected to automated separation and detection of
fragments on ABI 377 XL 96 lane sequencers. Initial sequence

contig sequence and gaps were filled in by primer walking reads
on the target DNA or by direct sequencing of PCR products. The
ends of the virus turned out to be absent in the shotgun
library, most probably due to cloning difficulties resulting

5   from the amino acids of pTP that remain bound to the ITR
sequences after proteinase K digestion of the viral DNA.
Additional sequence runs on viral DNA solved most of the
sequence in those regions, however it was difficult to obtain a
clear sequence of the most terminal nucleotides. At the 5' end

10 the sequence obtained was 5'-CCAATAATATACCT ..-3' while at the
3' end the obtained sequence was 5'-...AGGTATATTATTGATGATGGG-3'.
Most human adenoviruses have a terminal sequence 5'-
CATCATCAATAATATACC-3'. In addition, a clone representing the 3'
end of the Ad35 DNA obtained after cloning the terminal 7 kb

15 Ad35 EcoRI fragment into pBr322 (see example 4) also turned out
to have the typical CATCATCAATAAT... sequence. Therefore, Ad35
may have the typical end sequence and the differences obtained
in sequencing directly on the viral DNA are due to artefacts
correlated with run-off sequence runs and the presence of

20 residual amino acids of pTP.
The total sequence of Ad35 with corrected terminal sequences
is given in Figure 6. Based sequence homology with Ad5
(genbank # M72360) and Ad7 (partial sequence Genbank #
X03000) and on the location of open reading frames, the

25 organization of the virus is identical to the general
organization of most human adenoviruses, especially the
subgroup B viruses. The total length of the genome is 34794
basepairs.

30   Example 7
*Construction of a plasmid-based vector system to generate
recombinant Ad35-based viruses.*

A functional plasmid-based vector system to generate
recombinant adenoviral vectors comprises the following

35 components:

                                 50

1. An adapter plasmid comprising a left ITR and packaging
   sequences derived from Ad35 and at least one restriction
   site for insertion of an heterologous expression cassette
   and lacking E1 sequences. Furthermore, the adapter
5   plasmid contains Ad35 sequences 3' from the E1B coding
   region including the pIX promoter and coding sequences
   enough to mediate homologous recombination of the adapter
   plasmid with a second nucleic acid molecule.

2. A second nucleic acid molecule, comprising sequences
10   homologous to the adapter plasmid, and Ad35 sequences
   necessary for the replication and packaging of the
   recombinant virus, that is early, intermediate and late
   genes that are not present in the packaging cell.

3. A packaging cell providing at least functional E1
15   proteins capable of complementing the E1 function of
   Ad35.

Other methods for the generation of recombinant adenoviruses
on complementing packaging cells are known in the art and
may be applied to Ad35 viruses without departing from the
20 invention. As an example, the construction of a plasmid
based system, as outlined above, is described in detail
below.

1) Construction of Ad35 adapter plasmids.

25 Hereto, the adapter plasmid pAdApt (Figure 7; described in
example 2) was first modified to obtain adapter plasmids
that contain extended polylinkers and that have convenient
unique restriction sites flanking the left ITR and the
adenovirus sequence at the 3' end to enable liberation of
30 the adenovirus insert from plasmid vector sequences.
Construction of these plasmids is described below in detail:
Adapter plasmid pAdApt (Example 2) was digested with SalI
and treated with Shrimp Alkaline Phosphatase to reduce
religation. A linker, composed of the following two
35 phosphorylated and annealed oligo's: ExSalPacF 5' - TCG ATG
GCA AAC AGC TAT TAT GGG TAT TAT GGG TTC GAA TTA ATT AA- 3';

and ExSalPacR 5' - TCG ATT AAT TAA TTC GAA CCC ATA ATA CCC
ATA ATA GCT GTT TGC CA- 3'; was directly ligated into the
digested construct, thereby replacing the SalI restriction
site by Pi-PspI, SwaI and PacI. This construct was named

5   pADAPT+ExSalPac linker. Furthermore, part of the left ITR of
pAdApt was amplified by PCR using the following primers:
PCLIPMSF: 5'- CCC CAA TTG GTC GAC CAT CAT CAA TAA TAT ACC
TTA TTT TGG -3' and pCLIPBSRGI: 5'- GCG AAA ATT GTC ACT TCC
TGT G - 3'. The amplified fragment was digested with MunI

10   and BsrGI and cloned into pAd5/Clip (see Example 2), which
was partially digested with EcoRI and after purification
digested with BsrGI, thereby re-inserting the left ITR and
packaging signal. After restriction enzyme analysis, the
construct was digested with ScaI and SgrAI and an 800 bp

15   fragment was isolated from gel and ligated into ScaI/SgrAI
digested pADAPT+ExSalPac linker. The resulting construct,
named pIPspSalAdapt, was digested with SalI,
dephosphorylated, and ligated to the phosphorylated
ExSalPacF/ExSalPacR doublestranded linker mentioned above. A

20   clone in which the PacI site was closest to the ITR was
identified by restriction analysis and sequences were
confirmed by sequence analysis. This novel pAdApt construct,
termed pIPspAdapt (Figure 8) thus harbors two ExSalPac
linkers containing recognition sequences for PacI, PI-PspI

25   and BstBI, which surround the adenoviral part of the
adenoviral adapter construct, and which can be used to
linearize the plasmid DNA prior to cotransfection with
adenoviral helper fragments.

30   In order to further increase transgene cloning permutations
a number of polylinker variants were constructed based on
pIPspAdapt. For this purpose pIPspAdapt was first digested
with EcoRI and dephosphorylated. A linker composed of the
following two phosphorylated and annealed oligo's:

35   Ecolinker+: 5' -AAT TCG GCG CGC CGT CGA CGA TAT CGA TAG CGG
CCG C -3' and Ecolinker-: 5' -AAT TGC GGC CGC TAT CGA TAT

CGT CGA CGG CGC GCC G -3' was ligated into this construct,
thereby creating restriction sites for AscI, SalI, EcoRV,
ClaI and NotI. Both orientations of this linker were
obtained and sequences were confirmed by restriction

5   analysis and sequence analysis. The plasmid containing the
polylinker in the order 5' HindIII, KpnI, AgeI, EcoRI, AscI,
SalI, EcoRV, ClaI, NotI, NheI, HpaI, BamHI and XbaI was
termed pIPspAdapt1 (Figure 9) while the plasmid containing
the polylinker in the order HindIII, KpnI, AgeI, NotI, ClaI,

10   EcoRV, SalI, AscI, EcoRI, NheI, HpaI, BamHI and XbaI was
termed pIPspAdapt2.
To facilitate the cloning of other sense or antisense
constructs, a linker composed of the following two
oligonucleotides was designed, to reverse the polylinker of

15   pIPspAdapt: HindXba+ 5'-AGC TCT AGA GGA TCC GTT AAC GCT AGC
GAA TTC ACC GGT ACC AAG CTT A-3'; HindXba- 5'-CTA GTA AGC
TTG GTA CCG GTG AAT TCG CTA GCG TTA ACG GAT CCT CTA G-3'.
This linker was ligated into HindIII/XbaI digested
pIPspAdapt and the correct construct was isolated.

20   Confirmation was done by restriction enzyme analysis and
sequencing. This new construct, pIPspAdaptA, was digested
with EcoRI and the above mentioned Ecolinker was ligated
into this construct. Both orientations of this linker were
obtained, resulting in pIPspAdapt3 (Figure 10), which

25   contains the polylinker in the order XbaI, BamHI, HpaI,
NheI, EcoRI, AscI, SalI, EcoRV, ClaI, NotI, AgeI, KpnI and
HindIII. All sequences were confirmed by restriction enzyme
analysis and sequencing.

30   Adapter plasmids based on Ad35 were then constructed as
follows:
The left ITR and packaging sequence corresponding to Ad35 wt
sequences nucleotides 1 to 464 (Figure 6) were amplified by
PCR on wtAd35 DNA using the following primers:

35   Primer 35F1:

5'-CGG AAT TCT TAA TTA ATC GAC ATC ATC AAT AAT ATA CCT TAT AG-3'

Primer 35R2:

5'-GGT GGT CCT AGG CTG ACA CCT ACG TAA AAA CAG-3'

5   Amplification introduces a PacI site at the 5' end and an AvrII site at the 3' end of the sequence.

For the amplification Platinum Pfx DNA polymerase enzyme (LTI) was used according to manufacturers instructions but with primers at 0.6 μM and with DMSO added to a final

10 concentration of 3%. Amplification program was as follows: 2 min. at 94°C, (30 sec. 94°C, 30 sec. at 56°C, 1 min. at 68°C) for 30 cycles, followed by 10 min. at 68°C.

The PCR product was purified using a pcr purification kit (LTI) according to the manufacturers instructions and

15 digested with PacI and AvrII. The digested fragment was then purified from gel using the geneclean kit (Bio 101, Inc.)'.

The Ad5-based adapter plasmid pIPspAdApt-3 (Figure 10) was digested with AvrII and then partially with PacI and the 5762 bp fragment was isolated in an LMP agarose gel slice

20 and ligated with the abovementioned PCR fragment digested with the same enzymes and transformed into electrocompetent DH10B cells (LTI). The resulting clone is named pIPspAdApt3-Ad35lITR.

In parallel, a second piece of Ad35 DNA was amplified using

25 the following primers:

35F3: 5'- TGG TGG AGA TCT GGT GAG TAT TGG GAA AAC-3'
35R4: 5'- CGG AAT TCT TAA TTA AGG GAA ATG CAA ATC TGT GAG G-3'

The sequence of this fragment corresponds to nucl. 3401 to

30 4669 of wtAd35 (Figure 6) and contains 1.3 kb of sequences starting directly 3' from the E1B 55k coding sequence. Amplification and purification was done as described above for the fragment containing the left ITR and packaging sequence. The PCR fragment was then digested with PacI and

35 subcloned into pNEB193 vector (New England Biolabs) digested with SmaI and PacI. The integrity of the sequence of the

resulting clone was checked by sequence analysis. pNEB/Ad35pF3R4 was then digested with BglII and PacI and the Ad35 insert was isolated from gel using the QIAExII kit (Qiagen). pIPspAdApt3-Ad35lITR was digested with BglII and

5   then partially with PacI. The 3624 bp fragment (containing vector sequences, the Ad35 ITR and packaging sequences as well as the CMV promoter, multiple cloning region and polyA signal), was also isolated using the QIAExII kit (Qiagen). Both fragments were ligated and transformed into competent

10   DH10B cells (LTI). The resulting clone, pAdApt35IP3 (Figure 11), has the expression cassette from pIPspAdApt3 but contains the Ad35 left ITR and packaging sequences and a second fragment corresponding to nucl. 3401 to 4669 from Ad35. A second version of the Ad35 adapter plasmid having

15   the multiple cloning site in the opposite orientation was made as follows:

pIPspAdApt1 (Figure 9) was digested with NdeI and BglII and the 0.7 kbp band containing part of the CMV promoter, the MCS and SV40 polyA was isolated and inserted in the

20   corresponding sites of pAdApt35IP3 generating pAdApt35IP1 (Figure 12).

pAdApt35.LacZ and pAdApt35.Luc adapter plasmids were then generated by inserting the transgenes from pcDNA.LacZ (digested with KpnI and BamHI) and pAdApt.Luc (digested with

25   HindIII and BamHI) into the corresponding sites in pAdApt35IP1. The generation of pcDNA.LacZ and pAdApt.Luc is described in WO99/55132.

2) Construction of cosmid pWE.Ad35.pXI-rITR

30   Figure 13 presents the various steps undertaken to construct the cosmid clone containing Ad35 sequences from bp 3401 to 34794 (end of the right ITR) that are described in detail below.

A first PCR fragment (pIX-NdeI) was generated using the

35   following primer set:

35F5: 5'-CGG AAT TCG CGG CCG CGG TGA GTA TTG GGA AAA C -3'

35R6: 5'-CGC CAG ATC GTC TAC AGA ACA G-3'

DNA polymerase Pwo (Roche) was used according to manufacters instructions, however, with an endconcentration of 0.6 μM of both primers and using 50 ngr wt Ad35 DNA as template.

5   Amplification was done as follows: 2 min. at 94 °C, 30 cycles of 30 sec. at 94 °C, 30 sec. at 65 °C and 1 min. 45 sec. at 72 °C, followed by 8 min. at 68 °C. To enable cloning in the TA cloning vector PCR2.1, a last incubation with 1 unit superTaq polymerase (HT Biotechnology LTD) for

10 10 min. at 72 °C was performed.
The 3370 bp amplified fragment contains Ad35 sequences from bp 3401 to 6772 with a NotI site added to the 5' end. Fragments were purified using the PCR purification kit (LTI).

15 A second PCR fragment (NdeI-rITR) was generated using the following primers:
35F7: 5'-GAA TGC TGG CTT CAG TTG TAA TC -3'
35R8: 5'- CGG AAT TCG CGG CCG CAT TTA AAT CAT CAT CAA TAA TAT ACC-3'

20 Amplification was done with pfx DNA polymerase (LTI) according to manufacturer's instructions but with 0.6 μM of both primers and 3% DMSO using 10 ngr. of wtAd35 DNA as template. The program was as follows:
3 min. at 94 °C and 5 cycles of 30 sec. at 94 °C, 45 sec. at

25 40 °C, 2 min.45 sec. at 68 °C followed by 25 cycles of 30 sec. at 94 °C, 30 sec. at 60 °C, 2 min.45 sec. at 68 °C. To enable cloning in the TA-cloning vector PCR2.1, a last incubation with 1 unit superTaq polymerase for 10 min. at 72 °C was performed. The 1.6 kb amplified fragment ranging from

30 nucl. 33178 to the end of the right ITR of Ad35, was purified using the PCR purification kit (LTI).
Both purified PCR fragments were ligated into the PCR2.1 vector of the TA-cloning kit (Invitrogen) and transformed into STBL-2 competent cells (LTI). Clones containing the

35 expected insert were sequenced to confirm correct amplification. Next, both fragments were excised from the

vector by digestion with NotI and NdeI and purified from gel
using the geneclean kit (BIO 101, Inc.). Cosmid vector pWE15
(Clontech) was digested with NotI, dephosphorylated and also
purified from gel. These three fragments were ligated and

5   transformed into STBL2 competent cells (LTI). One of the
correct clones that contained both PCR fragments was then
digested with NdeI and the linear fragment was purified from
gel using the geneclean kit. Ad35 wtDNA was digested with
NdeI and the 26.6 kb fragment was purified from LMP gel

10   using agarase enzym (Roche) according to the manufacturers
instructions. These fragments were ligated together and
packaged using λ phage packaging extracts (Stratagene)
according to the manufacturer's protocol. After infection
into STBL-2 cells, colonies were grown on plates and

15   analyzed for presence of the complete insert. One clone with
the large fragment inserted in the correct orientation and
having the correct restriction patterns after independent
digestions with three enzymes (NcoI, PvuII and ScaI) was
selected. This clone is named pWE.Ad35.pIX-rITR. It contains

20   the Ad35 sequences from bp 3401 to the end and is flanked by
NotI sites (Figure 14).

3) Generation of Ad35 based recombinant viruses on PER.C6.

Wild type Ad35 virus can be grown on PER.C6 packaging

25   cells to very high titers. However, whether the Ad5-E1
region that is present in PER.C6 is able to complement E1-
deleted Ad35 recombinant viruses is unknown. To test this,
PER.C6 cells were cotransfected with the above described
adapter plasmid pAdApt35.LacZ and the large backbone

30   fragment pWE.Ad35.pIX-rITR. First, pAdApt35.LacZ was
digested with PacI and pWE.Ad35.pIX-rITR was digested with
NotI. Without further purification 4 μgr of each construct
was mixed with DMEM (LTI) and transfected into PER.C6 cells,
seeded at a density of 5×10^6 cells in a T25 flask the day

35   before, using Lipofectamin (LTI) according to the

manufacturers instructions. As a positive control, 6μgr of
PacI digested pWE.Ad35.pIX-rITR DNA was cotransfected with a
6.7 kb NheI fragment isolated from Ad35 wt DNA containing
the left end of the viral genome including the E1 region.

5   The next day medium (DMEM with 10% FBS and 10mM MgCl₂) was
refreshed and cells were further incubated. At day 2
following the transfection, cells were trypsinized and
transferred to T80 flasks. The positive control flask showed
CPE at five days following the transfection, showing that

10   the pWE.Ad35.pIX-rITR construct is functional at least in
the presence of Ad35-E1 proteins. The transfection with the
Ad35 LacZ adapter plasmid and pWE.Ad35.pIX-rITR did not give
rise to CPE. These cells were harvested in the medium at day
10 and freeze/thawed once to release virus from the cells. 4

15   ml of the harvested material was added to a T80 flask with
PER.C6 cells (at 80% confluency) and incubated for another
five days. This harvest/re-infection was repeated for two
times but there was no evidence for virus associated CPE.
From this experiment it seems that the Ad5-E1 proteins are

20   not, or not well enough, capable of complementing Ad35
recombinant viruses, however, it may be that the sequence
overlap of the adapter plasmid and the pWE.Ad35.pIX-rITR
backbone plasmid is not large enough to efficiently
recombine and give rise to a recombinant virus genome. The

25   positive control transfection was done with a 6.7 kb left
end fragment and therefore the sequence overlap was about
3.5 kb. The adapter plasmid and the pWE.Ad35.pIX-rITR
fragment have a sequence overlap of 1.3 kb. To check whether
the sequence overlap of 1.3 kb is too small for efficient

30   homologous recombination, a cotransfection was done with
PacI digested pWE.Ad35.pIX-rITR and a PCR fragment of Ad35
wtDNA generated with the above mentioned 35F1 and 35R4 using
the same procedures as described before. The PCR fragment
thus contains left end sequences up to bp 4669 and therefore

35   has the same overlap sequences with pWE.Ad35.pIX-rITR as the
adapter plasmid pAdApt35.LacZ but has Ad35 E1 sequences.

Following PCR column purification, the DNA was digested with SalI to remove possible intact template sequences. A transfection with the digested PCR product alone served as a negative control. Four days after the transfection, CPE

5   occurred in the cells transfected with the PCR product and the Ad35 pIX-rITR fragment, and not in the negative control. This shows that 1.3 kb overlapping sequences is sufficient to generate viruses in the presence of Ad35 E1 proteins. From these experiments we conclude that the presence of at

10   least one of the Ad35.E1 proteins is necessary to generate recombinant Ad35 based vectors from plasmid DNA on Ad5 complementing cell lines.

Example 8

15   *1) Construction of Ad35.E1 expression plasmids*

Since Ad5-E1 proteins in PER.C6 are not capable of complementing Ad35 recombinant viruses efficiently , Ad35 E1 proteins have to be expressed in Ad5 complementing cells (e.g. PER.C6) or a new packaging cell line expressing Ad35

20   E1 proteins has to be made, starting from either diploid primary human cells or established cell lines not expressing adenovirus E1 proteins. To address the first possibility, the Ad35 E1 region was cloned in expression plasmids as described below.

25   First, the Ad35 E1 region from bp 468 to bp 3400 was amplified from wtAd35 DNA using the following primer set:
35F11: 5'-GGG GTA CCG AAT TCT CGC TAG GGT ATT TAT ACC-3'
35F10: 5'-GCT CTA GAC CTG CAG GTT AGT CAG TTT CTT CTC CAC TG-3'

30   This PCR introduces a KpnI and EcoRI site at the 5' end and a SbfI and XbaI site at the 3' end.
Amplification on 5 ngr. template DNA was done with Pwo DNA polymerase (Roche) using manufacturers instructions, however, with both primers at a final concentration of 0.6

35   μM. The program was as follows: 2 min. at 94 °C, 5 cycles of 30 sec. at 94 °C, 30 sec. at 56 °C and 2 min. at 72 °C,

followed by 25 cycles of 30 sec. at 94°C, 30 sec. at 60 °C
and 2 min. at 72 °C, followed by 10 min. at 72 °C. PCR
product was purified by a PCR purification kit (LTI) and
digested with KpnI and XbaI. The digested PCR fragment was
5   then ligated to the expression vector pRSVhbvNeo (see
below), also digested with KpnI and XbaI. Ligations were
transformed into competent STBL-2 cells (LTI) according to
manufacturers instructions and colonies were analysed for
the correct insertion of Ad35E1 sequences into the
10 polylinker in between the RSV promoter and HBV polyA.
The resulting clone was named pRSV.Ad35-E1 (Figure 15). The
Ad35 sequences in pRSV.Ad35-E1 were checked by sequence
analysis.
    pRSVhbvNeo was generated as follows: pRc-RSV (Invitrogen)
15 was digested with PvuII, dephosphorylated with TSAP enzyme
(LTI) and the 3 kb vector fragment was isolated in low
melting point agarose (LMP). Plasmid pPGKneopA (Figure 16;
described in WO96/35798, was digested with SspI completely
to linearise the plasmid and facilitate partial digestion
20 with PvuII. Following the partial digestion with PvuII, the
resulting fragments were separated on a LMP agarose gel and
the 2245 bp PvuII fragment, containing the PGK promoter,
neomycine resistance gene and HBVpolyA, was isolated. Both
isolated fragments were ligated to give the expression
25 vector pRSV-pNeo that now has the original SV40prom-neo-
SV40polyA expression cassette replaced by a PGKprom-neo-
HBVpolyA cassette (Figure 17). This plasmid was further
modified to replace the BGHpA with the HBVpA as follows:
pRSVpNeo was linearised with ScaI and further digested with
30 XbaI. The 1145 bp fragment, containing part of the Amp gene
and the RSV promoter sequences and polylinker sequence, was
isolated from gel using the GeneClean kit (Bio Inc. 101).
Next pRSVpNeo was linearised with ScaI and further digested
with EcoRI partially and the 3704 bp fragment containing the
35 PGKneo cassette and the vector sequences were isolated from
gel as above. A third fragment, containing the HBV polyA

sequence flanked by XbaI and EcoRI at the 5' and 3' end
respectively, was then generated by PCR amplification on
pRSVpNeo using the following primer set:
HBV-F: 5'- GGC TCT AGA GAT CCT TCG CGG GAC GTC -3' and

5   HBV-R: 5'- GGC GAA TTC ACT GCC TTC CAC CAA GC -3'.
Amplification was done with Elongase enzyme (LTI) according
to the manufacturers instructions with the following
conditions: 30 seconds at 94°C, then 5 cycles of 45 seconds
at 94 °C, 1 minute at 42 °C and 1 minute 68 °C, followed by

10   30 cycles of 45 seconds at 94 °C, 1 minute at 65 °C and 1
minute at 68 °C, followed by 10 minutes at 68 °C. The 625 bp
PCR fragment was then purified using the Qiaquick PCR
purification kit, digested with EcoRI and XbaI and purified
from gel using the Geneclean kit. The three isolated

15   fragments were ligated and transformed into DH5α competent
cells (LTI) to give the construct pRSVhbvNeo (Figure 18). In
this construct the transcription regulatory regions of the
RSV expression cassette and the neomycine selection marker
are modified to reduce overlap with adenoviral vectors that

20   often contain CMV and SV40 transcription regulatory
sequences.

2) Generation of Ad35 recombinant viruses on PER.C6 cells cotransfected with an Ad35-E1 expression construct.

25   PER.C6 cells were seeded at a density of 5x10^6 cells in
a T25 flask and the next day transfected with a DNA mixture
containing:
          -      1 μg pAdApt35.LacZ digested with PacI
          -      5 μg pRSV.Ad35E1 undigested

30   -   2 μg pWE.Ad35.pIX-rITR digested with NotI
Transfection was done using Lipofectamine according to the
manufacturers instructions. Five hours after addition of the
transfection mixture to the cells, medium was removed and
replaced by fresh medium. After two days cells were

35   transferred to T80 flasks and further cultured. One week
post-transfection 1 ml of the medium was added to A549 cells

and the following day cells were stained for LacZ
expression. Blue cells were clearly visible after two hours
of staining indicating that recombinant LacZ expressing
viruses were produced. The cells were further cultured but
5   no clear appearance of CPE was noted. However, after 12 days
clumps of cells appeared in the monolayer and 18 days
following transfection cells were detached. Cells and medium
were then harvested, freeze-thawed once and 1 ml of the
crude lysate was used to infect PER.C6 cells in a 6-well
10   plate. Two days after infection cells were stained for LacZ
activity. After two hours 15% of the cells were stained
blue. To test for the presence of wt and / or replicating
competent viruses, A549 cells were infected with these
viruses and further cultured. No signs of CPE were found
15   indicating the absence of replication competent viruses.
These experiments show that recombinant AdApt35.LacZ viruses
were made on PER.C6 cells cotransfected with an Ad35-E1
expression construct.

20   3) Ad35 recombinant viruses escape neutralization in human
        serum containing neutralizing activity to Ad5 viruses.
The AdApt35.LacZ viruses were then used to investigate
infection in the presence of serum that contains
neutralizing activity to Ad5 viruses. Purified Ad5-based
25   LacZ virus served as a positive control for NA. Hereto,
PER.C6 cells were seeded in a 24-wells plate at a density of
$2x10^5$ cells/well. The next day a human serum sample with
high neutralizing activity to Ad5 was diluted in culture
medium in five steps of five times dilutions. 0.5 ml of
30   diluted serum was then mixed with $4x10^6$ virus particles
AdApt5.LacZ virus in 0.5 ml medium and after 30 minutes of
incubation at 37 °C, 0,5 ml of the mixture was added to
PER.C6 cells in duplicate. For the AdApt35.LacZ viruses, 0.5
ml of the diluted serum samples were mixed with 0.5 ml crude
35   lysate containing AdApt35.LacZ virus and after incubation
0.5 ml of this mixture was added to PER.C6 cells in duplo.

Virus samples incubated in medium without serum was used as
a positive control for infection. After two hours of
infection at 37 °C, medium was added to reach a final volume
of 1 ml and cells were further incubated. Two days after

5   infection cells were stained for LacZ activity. The results
are shown in Table II. From these results it is clear that
whereas AdApt5.LacZ viruses are efficiently neutralized,
AdApt35.LacZ viruses remain infectious irrespective of the
presence of human serum. This proofs that recombinant Ad35-

10   based viruses escape neutralization in human sera that
contain NA to Ad5-based viruses.

Example 9:

15

An Ad5/fiber35 chimeric vector with cell type specificity
for hemopoietic CD34 Lin stem cells
In example 3 we have described the generation of a library
of Ad5 based adenoviruses harboring fiber proteins of other

20   serotypes. As a non-limiting example for the use of this
library we here describe the identification of fiber-
modified adenoviruses that show improved infection of
hemopoietic stem cells.
Cells isolated from human bone marrow, umbilical cord blood,

25   or mobilized pheripheral blood carrying the flow cytometric
phenotype of being positive for the CD34 antigen and
negative for the early differentiation markers CD33, CD38,
and CD71 (lin⁻) are commonly referred to as hemopoietic stem
cells (HSC). Genetic modification of these cells is of major

30   interest since all hemopoietic lineages are derived from
these cells and therefore the HSC is a target cell for the
treatment of many acquired or congenital human hemopoietic
disorders. Examples of diseases that are amendable for
genetic modification of HSC, but not limited to, include

35   Hurlers disease, Hunters disease, Sanfilippos disease,
Morquios disease, Gaucher disease, Farbers disease, Niemann-

Pick disease, Krabbe disease, Metachromatic Leucodistrophy, I-cell disease, severe immunodeficiency syndrome, Jak-3 deficiency, Fucosidose deficiency, thallasemia, and erythropoietic porphyria. Besides these hemopoietic
5   disorders also strategies to prevent or treat aquired immunodeficiency syndrome (AIDS) and hemopoietic cancers are based on the genetic modification of HSCs or cells derived from the HSCs such as CD4 positive T lymphocytes in case of AIDS. The examples listed above thus aim at introducing DNA
10 into the HSC in order to complement on a genetic level for a gene and protein deficiency. In case of strategies for AIDS or cancer, the DNA to be introduced into the HSC can be anti-viral genes or suicide genes.
    Besides the examples listed above, there are several other
15 areas in which efficient transduction of HSCs using adenoviral vectors plays an important role. For instance in the field of tissue engeneering. In this area it is important to drive differentiation of HSCs to specific lineages. Some, non-limiting, examples are *ex vivo* bone
20 formation, cartilage formation, skin formation, as well as the generation of T-cell precursors or endothelial cell precursors. The generation of bone, cartilage or skin in bioreactors can be used for transplantation after bone fractures or spinal cord lessions or severe burn injuries.
25 Naturally, transduced cells can also directly be re-infused into a patient. The formation of large numbers of endothelial cell precursor from HSCs is of interest since these endothelial precursos cells can home, after re-infusion, to sites of cardiovascular injury such as
30 ischemia. Likewise, the formation of large numbers of T-cells from HSCs is of interest since these T-cell precursors can be primed, ex vivo, to eradicate certain targets in the human body after reinfusion of the primed T-cells. Preferred targets in the human body can be tumours or virus infected
35 cells.

64

From the examples described above, it can be concluded that
efficient gene delivery to HSCs is a major interest for the
field of gene therapy. Therefore, alteration of the
adenovirus serotype 5 host cell range to be able to target

5　　HSCs *in vitro* as well as *in vivo* is a major interest of the
invention. To identify a chimeric adenovirus with preferred
infection characteristics for human HSCs, we generated a
library of Ad5 based viruses carrying the fiber molecule
from alternative serotypes (serotypes 8, 9, 13, 16, 17, 32,

10　　35, 45, 40-L, 51). The generation of this fiber modified
library is described in example 3. Ad5 was taken along as a
reference. A small panel of this library was tested on human
TF-1 (erythroidleukemia, ATCC CRL-2003) whereas all
chimaeric viruses generated were tested on human primary

15　　stroma cells and human HSCs. Human TF-1 cell were routinly
maintained in DMEM suplemented with 10% FCS and 50 ng/ ml
IL-3 (Sandoz, Basel, Switzerland). Human primary fibroblast-
like stroma, isolated from a bone marrow aspirate, is
routinly maintained in DMEM/ 10% FCS. Stroma was seeded at a

20　　concentration of 1×10^5 cells per well of 24-well plates. 24
hours after seeding cells were exposed for 2 hours to 1000
virus particles per cell of Ad5, Ad5.Fib16, Ad5.Fib17,
Ad5.Fib35, Ad5.Fib40-L, or Ad5.Fib51 all carrying the green
fluorescent protein (GFP) as a marker. After 2 hours cells

25　　were washed with PBS and reseeded in medium without addition
of virus. TF-1 cells were seeded at a concentration of 2×10^5
cells per well of 24-well plates and were also exposed for 2
hours to 1000 virus particles of the different chimeric
adenoviruses. Virus was removed by washing the cells after

30　　the 2 hours exposure. Both cell types were harvested 48
hours after virus exposure and analysed for GFP expression
using a flow cytometer. The results on TF-1 cells, shown in
figure 19, demonstrates that chimeric adenoviruses carrying
a fiber from serotypes 16, 35, or 51 (all derived from

35　　adenovirus subgroup B) have preferred infection
characteristics as compared to Ad5 (subgroup C), Ad5.Fib17

(subgroup D), or Ad5.Fib40-L (subgroup F). Primary human
stroma was tested since these cells are commonly used as a
"feeder" cell to allow proliferation and maintenance of HSCs
under *ex vivo* culture conditions. In contrast to the

5   transduction of TF-1 cells, none of the fiber chimeric
adenoviruses were able to efficiently transduce human
primary stroma (Figure 20). Reasonable infection of human
fibroblast-like primary stroma was observed only with Ad5
despite the observation that none of the known receptor

10 molecules are expressed on these cells (see table III). The
absence of infection of human stroma using the chimeric
viruses is advantageous since in a co-culture setting, the
chimeric adenovirus will not be absorbed primarily by the
stroma "feeder" cells.

15

To test the transduction capacity of the fiber chimaeric
viruses, a pool of umbilical cord blood (3 individuals) was
used for the isolation of stem cells. CD34$^+$ cells were
isolated from mononuclear cell preparation using a MACS

20 laboratory separation system (Miltenyi Biotec) using the
protocol supplied by the manufacturer. Of the CD34$^+$ cells,
$2x10^5$ were seeded in a volume of 150 μl DMEM (no serum;
Gibco, Gaitherburg, MD) and 10 μl of chimeric adenovirus (to
give a final virus particles/cell ratio of 1000) was added.

25 The chimeric adenoviruses tested were Ad5, Ad5.Fib16,
Ad5.Fib35, Ad5Fib17, Ad5.Fib51 all containing Green
fluorescent protein (GFP) as a marker. Cells were incubated
for 2 hours in a humidified atmosphere of 10% CO_2 at 37^{0}C.
Thereafter, cells were washed once with 500 μl DMEM and

30 resuspended in 500 μl of StemPro-34 SF medium (Life
Technologies, Grand Island, NY).
Cells were then cultured for 5 days in 24-well plates
(Greiner, Frickenhausen, Germany) on irradiated (20 Gy) pre-
established human bone marrow stroma (ref 1), in a

35 humidified atmosphere of 10% CO2 at 37^{0}C. After 5 days, the
entire cell population was collected by trypsinization with

100 μl 0.25% Trypsin-EDTA (Gibco). The number of cells
before and after 5 days of culture was determined using a
hematocytometer. The number of CD34$^+$ and CD34^{++}CD33,38,71$^-$
cells in each sample was calculated from the total number of
5   cells recovered and the frequency of the CD34^{++}CD33,38,71$^-$
cells in the whole population as determined by FACS
analysis. The transduction efficiency was determined by FACS
analysis while monitoring in distinct sub populations the
frequency of GFP expressing cells as well as the intensity
10   of GFP per individual cell. The results of this experiment,
shown in figure 21, demonstrates that adenovirus serotype 5
or the chimeric adenovirus Ad5.Fib17 does not infect
CD34$^+$Lin$^-$ cells as witnessed by the absence of GFP
expression. In contrast, with the chimeric viruses carrying
15   the fiber molecule of serotypes 16, 51, or 35 high
percentages of GFP positive cells are scored in this cell
population. Specificity for CD34 Lin is demonstrated since
little GFP expression is observed in CD34$^+$ cells that are
also expressing CD33, CD38, and CD71. Subfractioning of the
20   CD34$^+$Lin$^-$ cells (Figure 22) showed that the percentage of
cells positive for GFP declines using Ad5.Fib16, Ad5.Fib35,
or Ad5.Fib51 when the cells become more and more positive
for the early differentiation markers CD33 (myeloid), CD71
(erythroid), and CD38 (common early differentiation marker).
25   These results thus demonstrate the specificity of the
chimeric adenoviruses Ad5.Fib16, Ad5.Fib35, and Ad5.Fib51
for HSCs. Figure 23 shows an alignment of the Ad5 fiber with
the chimeric B-group fiber proteins derived from Ad16, 35
and 51.By determining the number of cells recovered after
30   the transduction procedure the toxicity of adenovirus can be
determined. The recovery of the amount of CD34+ cells as
well as the amount of CD34$^+$Lin$^-$ (Figure 24) demonstrates
that a 2 hour exposure to 1000 adenovirus particles did not
have an effect on the number of cells recovered.
35

Example 10

An Ad5/fiber35 chimeric vector with cell type specificity for Dendritic cells

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Dendritic cells are antigen presenting cells (APC), specialized to initiate a primary immune response and able to boost a memory type of immune response. Dependent on their stage of development, DC display different functions:
10 immature DC are very efficient in the uptake and processing of antigens for presentation by Major Histocompatibility Complex (MHC) class I and class II molecules, whereas mature DC, being less effective in antigen capture and processing, perform much better at stimulating naive and memory CD4$^+$ and
15 CD8$^+$ T cells, due to the high expression of MHC molecules and co-stimulatory molecules at their cell surface. The immature DCs mature *in vivo* after uptake of antigen, travel to the T-cell areas in the lymphoid organs, and prime T-cell activiation.
20 Since DCs are the cells responsible for triggering an immune response there has been a long standing interest in loading DCs with immunostimulatory proteins, peptides or the genes encoding these proteins to trigger the immune system. The applications for this strategy are in the field of cancer
25 treatment as well as in the field of vaccination. So far, anti-cancer strategies have focussed primarily on *ex vivo* loading of DCs with antigen (protein or peptide). These studies have revealed that this procedure resulted in in induction of cytotoxic T cell activity. The antigens used to
30 load the cells are generally identified as being tumor specific. Some, non-limiting, examples of such antigens are GP100, mage, or Mart-1 for melanoma.

Besides treatment of cancer many other potential human
35 diseases are currently being prevented through vaccination. In the vaccination strategy, a "crippled" pathogen is

presented to the immune system via the action of the antigen
presenting cells, i.e. the immature DCs. Well-known examples
of disease prevention via vaccination strategies include
Hepatitis A,B, and C, influenza, rabies, yellow fever,
5   measles. Besides these well-known vaccination programs,
research programs for treatment of malaria, ebola, river
blindness, HIV and many other diseases are being developed.
Many of the above mentioned pathogens are considered to
dangerous for the generation of a "crippled" pathogen
10   vaccine. This latter thus calls for the isolation and
characterization of proteins of each pathogen which is able
to mount a "full blown" immune response thus resulting in
complete protection upon challenge with wild type pathogen.
For this strategy of loading DCs with immunostimulatory
15   proteins or peptides to become therapeutically feasible
At least two distinct criteria have to be met 1) the
isolation of large numbers of DCs which can be isolated,
manipulated, and reinfused into a patient, making the
procedure autologous. To date, it is possible to obtain such
20   large quantities of immature DCs from cultured peripheral
blood monocytes from any given donor. 2) a vector which can
transduce DCs efficiently such that the DNA encoding for an
immunostimulatory protein can be delivered. The latter is
extremely important since it has become clear that the time
25   required for DCs to travel to the lymphoid organs is such
that most proteins or peptides are already released from the
DCs resulting in incomplete immune priming. Because DCs are
terminally differentiated and thus non-dividing cells,
recombinant adenoviral vectors are are being considered for
30   delivering the DNA encoding for antigens to DCs. Ideally
this adenovirus should have a high affinity for dendritic
cells but also should not be recognized by neutralizing
antibodies of the host such that *in vivo* transduction of DCs
can be accomplished. This latter would omit the need for *ex*
35   *vivo* manipulations of DCs but would result in a medical

procedure identical to the vaccination programs which are
currently in place, i.e. intramuscular or subcutaneous
injection predominantly. Thus, DC transduced by adenoviral
vectors encoding an immunogenic protein may be ideally

5   suited to serve as natural adjuvants for immunotherapy and
vaccination
From the above described examples, it can be concluded that
efficient gene delivery to DCs is a major interest for the
field of gene therapy. Therefore, alteration of the

10   adenovirus serotype 5 host cell range to be able to target
DCs *in vitro* as well as *in vivo* is a major interest of the
invention. To identify a chimeric adenovirus with preferred
infection characteristics for human DCs, we generated a
library of Ad5 based viruses carrying the fiber molecule

15   from alternative serotypes (serotypes 8, 9, 13, 16, 17, 32,
35, 45, 40-L, 51). Ad5 was taken along as a reference.
We evaluated the susceptibility of human monocyte derived
immature and mature DC to recombinant chimeric adenoviruses
expressing different fibers.

20   Human PBMC from healthy donors were isolated through Ficoll-
Hypaque density centrifugation. Monocytes were isolated from
PBMC by enrichement for CD14+ cells using staining with FITC
labeled anti-human CD 14 monoclonal antibody (Becton
Dickinson), anti FITC microbeads and MACS separation columns

25   (Miltenyi Biotec).
This procedure usually results in a population of cells that
are < 90 % CD14+ as analysed by FACS. Cells were placed in
culture using RPMI-1640 medium (Gibco) containing 10% Foetal
Bovine Serum (Gibco), 200 ng/ml rhu GM-CSF (R&D/ITK

30   diagnostics, 100 ng/ml rhu IL-4 (R&D/ITK diagnostics) and
cultured for 7 days with feeding of the cultures with fresh
medium containing cytokines on alternate days. The immature
DC resulting from this procedure after 7 days express a
phenotype CD83-,CD14 low or CD14-,HLA-DR+, as was demonstrated

35   by FACS analysis. Immature DC are matured by culturing the

70

cells in medium containing 100 ng/ml TNF-a for 3 days, where after they expressed CD83 on their cell surface.

In a pilot experiment 5.10^5 immature DCs were seeded in wells of 24-well plates and exposed for 24 hours to 100 and

5 1000 virus particles per cell of each fiber recombinant virus. Virus tested was adenovirus serotype 5 (Ad5), and the fiber chimeric viruses based on Ad5: Ad5.Fib12, Ad5.Fib16, Ad5.Fib28, Ad5.Fib32, Ad5.Fib40-L (long fiber of serotype 40), Ad5.Fib49, and Ad5.Fib51 (where Fibxx stands for the

10 serotype of which the fiber molecule is derived). these viruses are derived from subgroup C, A, B, D, D, F, D, and B respectively. After 24-hours cells were lysed (1% Triton X-100/ PBS) and luciferase activity was determined using a protocol supplied by the manufacturer (Promega, Madison, WI,

15 USA). The results of this experiment, shown in figure 25, demonstrates that Ad5 poorly infects immature DCs as witnessed by the low level of transgene expression. In contrast, Ad5.Fib16 and Ad5.Fib51 (both a B-group fiber chimeric virus) and also Ad5.Fib40-L (Subgroup F) show

20 efficient infection of immature DCs based on luciferase transgene expression.

In a second experiment, 5.10^5 immature and mature DC were infected with 10000 virus particles per cell of Ad5, Ad5.Fib16, Ad5.Fib40-L, and Ad5.Fib51 all carrying the LacZ

25 gene as a marker. LacZ expression was monitored by flow cytometric analysis using a CM-FDG kit system and the instructions supplied by the manufacturer (Molecular probes, Leiden, The Netherlands). The results of this experiment, shown in figure 26, correlates with the previous experiment

30 in that Ad5.Fib16 and Ad5.Fib51 are superior to Ad5 in transducing mature and immature human DCs. Also, this experiment shows that Ad5.Fib40-L is not as good as Ad5.Fib16 and Ad5.Fib51 but better than Ad5.

Based on these results we tested other chimeric adenoviruses

35 containing fibers of B group viruses e.g. Ad5.Fib11 and Ad5.Fib35 for there capacity to infect DCs. We focussed on

immature DCs since these are the cells that process an
expressed transgene product into MHC class I and II
presentable peptides. Immature DC's were seeded at a cell
density of 5.10^5 cells/well in 24 well plates (Costar) and

5   infected with 1000 and 5000 virus particles per cell after
which the cells were cultured for 48 hours under conditions
for immature DCs prior to cell lysis and Luciferase activity
measurements. The result of this experiment, shown in figure
27, demonstrate that Ad5 based chimeric adenoviruses

10   containing fibers of group-B viruses efficiently infect
immature DCs. In a fourth experiment we again infected
immature DCs identically as described in the former
experiments but this time Ad5, Ad5.Fib16, and Ad5.Fib35 were
used carrying green fluorescent protein (GFP) as a

15   markergene. The results on GFP expression measured with a
flow cytometer 48 hours after virus exposure is shown in
figurte 28 and correlates with the data obtained so far.
Thus, the results so far are consistent in that Ad5 based
vectors carrying a fiber from a alternative adenovirus

20   derived from subgroup B predominantly fiber of 35, 51, 16,
and 11 are superior to Ad5 for transducing human DCs.
The adenoviruses disclosed herein are also very suitable in
vaccination of animals. To illustrate this, we tested DCs
derived from mouse and chimpanzee to identify whether these

25   viruses can be used in these animal models. This latter in
particular since the receptor for human adenovirus derived
from subgroup B is unknown to date and therefore it is
unknown whether this protein is conserved among species. For
both species immature DCs were seeded at a density of 10^5

30   cells per well of 24-well plates. Cells were subsequently
exposed for 48 hours to 1000 virus particles per cell of
Ad5, Ad5Fib16, and Ad5.Fib51 in case of mouse DC and Ad5,
and Ad.Fib35 in case of chimpanzee DCs (see figure 29). The
mouse experiment was performed with viruses carrying

35   luciferase as a marker and demonstrated approximately 10-50
fold increased luciferase activity as compared to Ad5. The

chimanzee DCs were infected with the GFP viruses and were
analysed using a flow cytometer. These results, also shown
in figure 29, demonstrate that Ad5 (3%) transduces
chimpanzee DCs very poorly as compared to Ad5.Fib35 (66.5%).

5

Example 11

*Construction of a plasmid-based vector system to generate
Ad11-based recombinant viruses*

The results of the neutralization experiments described in
10   Example 5 show that Ad11, like Ad35, was also not
neutralized in the vast majority of human serum samples.
Therefore, recombinant adenoviruses based on Ad11 are
preferred above the commonly used Ad2 and Ad5-based vectors
as vectors for gene therapy treatment and vaccination. Both
15   Ad35 and Ad11 are B-group viruses and are classified as
viruses belonging to DNA homology cluster 2 (Wadell, 1984).
Therefore, the genomes of Ad35 and Ad11 are very similar. To
generate a plasmid based system for the production of Ad11-
based recombinant viruses the adapter plasmid pAdApt35IP1
20   generated in Example 7 is modified as follows. Construct
pAdApt35IP1 is digested with AvrII and then partially with
PacI. The digestion mixture is separated on gel and the 4.4
kb fragment containing the expression cassette and the
vector backbone is isolated using the geneclean kit (BIO
25   101, Inc.). Then a PCR amplification is performed on wtAd11
DNA using the primers 35F1 and 35R2 (see Example 7) using
Pwo DNA polymerase according to the manufacturers
instructions. The obtained PCR fragment of 0.5 kb is
purified using the PCR purification kit (LTI) and ligated to
30   the above prepared fragment of pAdApt35IP1. This gives
construct pAdApt11-35IP1 in which the 5' adenovirus fragment
is exchanged for the corresponding sequence of Ad11. Next,
pAdApt11-35IP1 is digested with BglII and partially with
PacI. The obtained fragments are separated on gel and the
35   3.6 kb fragment containing the vector sequences, the 5'
adenovirus fragment and the expression cassette is purified

from gel as above. Next, a PCR fragment is generated using
primers 35F3 and 35R4 (see Example 7) on wtAd11 DNA.
Amplification is done as above and the obtained 1.3 kb
fragment is purified and digested with BglII and PacI. The
5   isolated fragments are then ligated to give construct
pAdApt11IP1. This adapter plasmid now contains Ad11
sequences in stead of Ad35 sequences. Correct amplification
of PCR amplified Ad11 sequences, is verified by comparison
of the sequence in this clone with the corresponding
10   sequence of Ad11 DNA. The latter is obtained by direct
sequencing on Ad11 DNA using the indicated PCR primers. The
large cosmid clone containing the Ad11 backbone is generated
as follows. First, a PCR fragment is amplified on Ad11 DNA
using the primers 35F5 and 35R6 with Pwo DNA polymerase as
15   described in Example 7 for Ad35 DNA. The PCR fragment is
then purified using the PCR purification kit (LTI) and
digested with NotI and NdeI. The resulting 3.1 kb fragment
is isolated from gel using the geneclean kit (Bio 101,
Inc.). A second PCR fragment is then generated on Ad11 DNA
20   using the primers 35F7 and 35R8 (see Example 7) with Pwo DNA
polymerase according to the manufacturers instructions and
purified using the PCR purification kit (LTI). This
amplified fragment is also digested with NdeI and NotI and
the resulting 1.6 kb fragment is purified from gel as above.
25   The two digested PCR fragments are then ligated together
with cosmid vector pWE15, previously digested with NotI and
dephosphorylated using Tsap enzyme (LTI) according to
manufacturers instructions. One clone is selected that has
one copy of both fragments inserted. Correct clones are
30   selected by analytical NotI digestion that gives a fragment
of 4.7 kb. Confirmation is obtained by a PCR reaction using
primers 35F5 and 35R8 that gives a fragment of the same
size. The correct clone is then linearized with NdeI and
isolated from gel. Next, wtAd11 DNA is digested with NdeI
35   and the large 27 kb fragment is isolated from Low melting
point agarose gel using agarase enzyme (Roche) according to

the manufacturers instructions. Both fragments are then
ligated and packaged using λ phage packaging extracts
(Stratagene) according to the manufacturers protocol. After
infection into STBL-2 cells (LTI) colonies are grown on
5   plates and analysed for the presence of the complete insert.
The functionality of selected clones is then tested by
cotransfection on PER.C6. Hereto, the DNA is digested with
NotI and 6 μgr is cotransfected with 2 μgr of a PCR fragment
generated on Ad11 DNA with primers 35F1 and 35R4 (see
10   example 7). Correct clones give CPE within one week
following transfection. The correct clone is named
pWE.Ad11.pIX-rITR.
Using the above described procedure, a plasmid-based system
consisting of an adapter plasmid suitable for insertion of
15   foreign genes and a large helper fragment containing the
viral backbone is generated. Recombinant Ad11-based viruses
are made using the methods described inhere for Ad35-based
recombinant viruses.

20

Example 12
*Neutralization of adenoviruses in samples derived from
patients*

25   In the neutralization experiments described in Examples
1 and 5, all samples were derived from healthy volunteers.
Since one of the applications of non-neutralized vectors is
in the field of gene therapy, it is interesting to
investigate whether Ad35 is also neutralized with a low
30   frequency and with low titers in groups of patients that are
candidates for treatment with gene therapy.

- Cardio-vascular disease patients
35   26 paired serum and pericardial fluid (PF) samples were
obtained from patients with heart faillure. These were

75

tested against Ad5 and Ad35 using the neutralization assay
described in Example 1. The results confirmed the previous
data with samples from healthy volunteers. 70% of the serum
samples contained NA to Ad5 and 4% to Ad35. In the

5   pericardial fluid samples the titers were lower resulting in
a total of 40% with NA to Ad5 and none to Ad35. There was a
good correlation between NA in PF and serum i.e. there were
no positive PF samples whithout NA in the paired serum
sample. These results show that non-neutralized vectors

10   based on Ad35 are preferred over Ad5 vectors for treatment
of cardio-vascular diseases. As is true for all forms of
non-neutralized vectors in this application, the vector may
be based on the genome of the non-neutralized serotype or
may be based on Ad5 (or another serotype) though displaying

15   at least the major capsid proteins (hexon, penton and
optionally fiber) of the non-neutralized serotype.

- Rheumatoid Arthritis patients
The molecular determinant underlying arthritis is not yet

20   known but both T-cell disfunction and imbalanced growth
factor production in joints is known to cause inflammation
and hyperplasia of synovial tissue. The synoviocytes start
to proliferate and invade the cartilage and bone which leads
to destruction of these tissues. Current treatment starts

25   (when in an early stage) with administration of anti-
inflammatory drugs (anti-TNF, IL1-RA, IL-10) and/or
conventional drugs (e.g. MTX, sulfasalazine). In late stage
RA synovectomy is performed which is based on surgery,
radiation, or chemical intervention. An alternative or

30   additional option is treatment via gene therapy where an
adenoviral vector is delivered directly into the joints of
patients and expresses an anti-inflammatory drug or a
suicide gene. Previous studies performed in rhesus monkeys
suffering from collagen-induced arthritis have shown that

35   Ad5-based vectors carrying a marker gene can transduce
synoviocytes. Whether in the human situation adenoviral

delivery is hampered by the presence of NA is not known. To investigate the presence of NA in synovial fluid (SF) of RA patients, SF samples were obtained from a panel of 53 random selected patients suffering from rheumatoid arthritis (RA).

5   These were tested against several wt adenoviruses using the neutralization assay as described in Example 1. Results of this screen are presented in Table III. Adenovirus type 5 was found to be neutralized in 72% of the SF samples. Most of these samples contain high titers of NA as also the

10   highest dilution of the SF sample that was tested (64x) neutralized Ad5 viruses. This means that adenoviral vector delivery to the synoviocytes in the joints of RA patients will be very inefficient. Moreover, since the titers in the SF are so high it is doubtfull whether lavage of the joints

15   prior to vector injection will remove enough of the NA. Of the other serotypes that were tested Ad35 was shown to be neutralized in only 4% of the samples. Therefore, these data confirm the results obtained in serum samples from healthy patients and show that for treatment of rheumatoid arthritis

20   Ad35-based vectors or chimeric vectors displaying at least some of the capsid proteins from Ad35 are preferred vectors.

Example 13

25   *Modifications in the backbone of Ad35-based viruses*

1) Generation of pBr/Ad35.Pac-rITR and pBr/Ad35.PRn
     Example 4 describes the generation of the Ad35 subclone
     pBr/Ad35.Eco13.3. This clone contains Ad35 sequences from bp

30   21943 to the end of the right ITR cloned into the EcoRI and EcoRV sites of pBr322. To extend these sequences to the PacI site located at bp 18137 in Ad35, pBr/Ad35.Eco13.3 (see Example 4) was digested with AatII and SnaBI and the large vector -containing fragment was isolated from gel using the

35   QIAEX II gel extraction kit (Qiagen). Ad35 wt DNA was digested with PacI and SnaBI and the 4.6 kb fragment was

isolated as above. This fragment was then ligated to a
double-stranded (ds) linker containing a PacI and an AatII
overhang. This linker was obtained after annealing the
following oligonucleotides:

5   A-P1: 5'-CTG GTG GTT AAT-3'
       A-P2: 5'-TAA CCA CCA GAC GT-3'
       The ligation mix containing the ds linker and the PacI-SnaBI
       Ad35 fragment was separated from unligated linker on a LMP
       gel. The 4.6 kb band was cut out the gel, molten at 65 °C,
10   and then ligated to the purified pBr/Ad35.Eco13.3 vector
       fragment digested with AatII and SnaBI. Ligations were
       transformed into electrocompetent DH10B cells (Life
       Technologies Inc.). The resulting clone, pBr/Ad35.Pac-rITR,
       contained Ad35 sequences from the PacI site at bp 18137 upto
15   the right ITR.
       Next, a unique restriction site was introduced at the 3' end
       or the right ITR to be able to free the ITR from vector
       sequences. Hereto, a PCR fragment was used that covers Ad35
       sequences from the NdeI site at bp 33165 to the right ITR
20   having the restriction sites SwaI, NotI and EcoRI attached
       to the rITR. The PCR fragment was generated using primers
       35F7 and 35R8 (described in example 7). After purification,
       the PCR fragment was cloned into the AT cloning vector
       (Invitrogen) and sequenced to verify correct amplification.
25   The correct amplified clone was then digested with EcoRI,
       blunted with Klenow enzym and subsequently digested with
       NdeI and the PCR fragment was isolated. In parallel, the
       NdeI in the pBr vector in pBr/Ad35.Pac-rITR was removed as
       follows: A pBr322 vector from which the NdeI site was
30   removed by digestion with NdeI, Klenow treatment and
       religation, was digested with AatII and NheI. The vector
       fragment was isolated in LMP gel and ligated to the 16.7 kb
       Ad35 AatII-NheI fragment from pBr/Ad35.Pac-rITR that was
       also isolated in an LMP gel. This generated pBr/Ad35.Pac-
35   rITR.ΔNdeI. Next pBr/Ad35.Pac-rITR.ΔNdeI was digested with
       NheI, the ends were filled in using Klenow enzym and the DNA

was then digested with NdeI. The large fragment containing
the vector and Ad35 sequences was isolated. Ligation of this
vector fragment and the PCR fragment resulted in
pBr/Ad35.PRn. In this clone specific sequences coding for

5   fiber, E2A, E3, E4 or hexon can be manipulated. In addition,
promoter sequences that drive for instance the E4 proteins
or the E2 can be mutated or deleted and exchanged for
heterologous promoters.

10 2) Generation of Ad35-based viruses with fiber proteins from
        different serotypes.
        Adenoviruses infect human cells with different
    efficiencies. Infection is accomplished by a two step
    process involving: 1. the fiber proteins that mediate

15 binding of the virus to specific receptors on the cells, and
    2. the penton proteins that mediate internalization by
    interaction of for example the RGD sequence to integrins
    present on the cell surface. For subgroup B viruses of which
    Ad35 is a member, the cellular receptor for the fiber

20 protein is not known. There are striking differences in
    infection efficiency of human cells of subgroup B viruses
    compared to subgroup C viruses like Ad5 (see WO 00/03029 and
    EP 99200624.7). Even within one subgroup infection
    efficiencies of certain human cells may differ between

25 various serotypes. For example, the fiber of Ad16, when
    present on an Ad5-based recombinant virus infects primary
    endothelial cells, smooth muscle cells and synoviocytes of
    human and rhesus monkey origin better than Ad5 chimeric
    viruses carrying the fiber of Ad35 or Ad51. Thus, to obtain

30 high infection efficiencies of Ad35-based viruses, it may be
    necessary to change the fiber protein for a fiber protein of
    a different serotype. The technology for such fiber chimeras
    is described for Ad5-based viruses in Example 3, and is
    below examplified for Ad35 viruses.

35 First, most fiber sequences are deleted from the Ad35
    backbone in construct pBr/Ad35.PRn as follows:

The left flanking sequences and part of the fiber protein in
Ad35 ranging from bp 30225 upstream of a unique MluI site up
to bp 30872 (numbers according to wt Ad35 sequence as
disclosed in Figure 6) in the tail of fiber are amplified

5 using primers
DF35-1 : 5'-CAC TCA CCA CCT CCA ATT CC-3'
and
DF35-2: 5'-CGG GAT CCC GTA CGG GTA GAC AGG GTT GAA GG-3'
This PCR amplification introduces an unique BsiWI site in

10 the tail of the fiber gene.
The right flanking sequences ranging from the end of the
fiber protein at bp 31798 to bp 33199 (numbering according
to wtAd35 sequence, Figure 6) , 3' from the unique NdeI site
is amplified using primers

15 DF35-3: 5'-CGG GAT CCG CTA GCT GAA ATA AAG TTT AAG TGT TTT
TAT TTA AAA TCA C-3'
ana
DF35-4: 5'-CCA GTT GCA TTG CTT GGT TGG-3'.
This PCR introduces a unique NheI site in the place of the

20 fiber sequences. PCR amplification is done with Pwo DNA
polymerase (Roche) according to the manufacturers
instructions. After amplification the PCR products are
purified using a PCR purification kit and the fragments are
digested with BamHI and ligated together. The 2 kb ligated

25 fragments are purified from gel and cloned in the PCR Script
Amp vector (Stratagene). Correct amplification is checked by
sequencing. The PCR fragment is then excised as a MluI/NdeI
fragment and cloned in pBr/Ad35.PRn digested with the same
enzymes. This generates pBr/Ad35.PRΔfib, a shuttle vector

30 suitable to introduce fiber sequences of alternative
serotypes. This strategy is analogous to the fiber
modification strategy for Ad5-based viruses as disclosed in
WO00/03029. Primers that are listed in Table I of that
application were used to amplify fiber sequences of various

35 subgroups of adenovirus. For amplification of fibers that
are cloned in the pBr/Ad35.PRΔfib the same (degenerate)

primer sequences can be used, however, the NdeI site in the
forward primers (tail oligonucleotides A to E) should be
changed to a BsiWI site and the NsiI site in the reverse
oligo (knob oligonucleotide 1 to 8) should be changed in a
5   NheI site. Thus fiber 16 sequences are amplified using the
following degenerate primers:
5'- CCK GTS TAC CCG TAC GAA GAT GAA AGC-3' and 5'-CCG GCT
AGC TCA GTC ATC TTC TCT GAT ATA-3'. Amplified sequences are
then digested with BsiWI and NheI and cloned into
10   pBr/Ad35.PRΔfib digested with the same enzymes to generate
pBr/Ad35.PRfib16. The latter construct is then digested with
PacI and SwaI and the insert is isolated from gel. The
PacI/SwaI Ad35 fragment with modified fiber is then cloned
into the corresponding sites of pWE/Ad35.pIX-rITR to give
15   pWE/Ad35.pIX-rITR.fib16. This cosmid backbone can then be
used with an Ad35-based adapter plasmid to generate Ad35
recombinant viruses that display the fiber of Ad16. Other
fiber sequences can be amplified with (degenerate) primers
as mentioned above. If one of the fibers sequences turns out
20   to have an internal BsiWI or NheI site, the PCR fragment has
to be digested partially with that enzyme.

3) Generation of Ad35-based viruses with inducible, E1
   independent, E4 expression.
25   The adenovirus E4 promoter is activated by expression of
E1 proteins. It is not known whether the Ad5 E1 proteins are
capable of mediating activation of the Ad35 E4 promoter.
Therefore, to enable production of Ad35 recombinant viruses
on PER.C6 cells, it may be advantageous to make E4
30   expression independent of E1. This can be achieved by
replacing the Ad35-E4 promoter by heterologous promoter
sequences like, but not limited to, the 7xTetO promoter.
Recombinant E1-deleted Ad5-based vectors are shown to have
residual expression of viral genes from the vector backbone
35   in target cells, despite the absence of E1 expression. Viral
gene expression increases the toxicity and may trigger a

host immune response to the infected cell. For most
applications of adenoviral vectors in the field of gene
therapy and vaccination it is desired to reduce or diminish
the expression of viral genes from the backbone. One way to

5   achieve this is to delete all, or as much as possible,
sequences from the viral backbone. By deleting E2A, E2B or
E4 genes and/or the late gene functions, one has to
complement for these functions during production. This
complementation can either be by means of a helper virus or

10   through stable addition of these functions, with or without
inducible transcription regulation, to the producer cell.
Methods to achieve this have been described for Ad5 and are
known in the art. One specific method is replacement of the
E4 promoter by promoter sequences that are not active in the

15   target cells. E4 proteins play a role in for example
replication of adenoviruses through activation of the E2
promoter and in late gene expression through regulation of
splicing and nuclear export of late gene transcripts. In
addition, at least some of the E4 proteins are toxic to

20   cells. Therefore, reduction or elimination of E4 expression
in target cells will further improve Ad35-based vectors. One
way to achieve this is to replace the E4 promoter by an
heterologous promoter that is inactive in the target cells.
An example of a heterologous promoter/activator system that

25   is inactive in target cells is the tetracyclin inducible
TetO system (Gossen and Bujard, 1992). Other prokaryotic or
synthetic promoter/activator systems may be used. In this
example, the E4 promoter in the backbone of the viral vector
is replaced by a DNA fragment containing 7 repeats of the

30   tetracyclin responsive element from the tet operon (7xTetO).
A strong transactivator for this promoter is a fusion
protein containing the DNA binding domain of the tet
repressor and the activation domain of VP16 (Tet
transactivator protein, Tta). Strong E4 expression,

35   independent of E1 expression, can be accomplished in PER.C6
cells expressing Tta. Tta expressing PER.C6 cells have been

generated and described (see Example 15). Ad5 derived E1-
deleted viruses with E4 under control of 7xTetO can be
generated and propagated on these cells. Following infection
in cells of human or animal origin (that do not express the
5   Tta transactivator), E4 expression was found to be greatly
diminished compared to E1 deleted viruses with the normal E4
promoter.
Below the construction of pWE/Ad35.pIX-rITR.TetO-E4, a
cosmid helper vector to produce viruses with the E4 promoter
10   replacement, is described.
First, a fragment was generated by PCR amplification on
pBr/Ad35.PRn DNA using the following primers:
355ITR: 5'- GAT CCG GAG CTC ACA ACG TCA TTT TCC CAC G-3'
and
15   353ITR: 5'-CGG AAT TCG CGG CCG CAT TTA AAT C-3'
This fragment contains sequences between bp 34656 (numbering
according to wtAd35) and the NotI site 3' of the right ITR
in pBr/Ad35.PRn and introduces an SstI site 5' of the right
ITR sequence.
20   A second PCR fragment was generated on pBr/Ad35.PRn DNA
using primers:
35DE4: 5'-CCC AAG CTT GCT TGT GTA TAT ATA TTG TGG-3' and
35F7: See example 7.
This PCR amplifies Ad35 sequences between bp 33098 and 34500
25   (numbering according to wtAd35) and introduces a HindIII
site upstream of the E4 Tata-box. With these two PCR
reactions the right- and left -flanking sequences of the E4
promoter are amplified. For amplification, Pwo DNA
polymerase was used according to manufacturers instructions
30   A third fragment containing the 7xTetO promoter was isolated
from construct pAAO-E-TATA-7xTetO by digestion with SstI and
HindIII. The generation of pAAO-E-TATA-7xTetO is described
below. The first PCR fragment (355/353) was then digested
with SstI and NotI and ligated to the 7xTetO fragment. The
35   ligation mixture was then digested with HindIII and NotI and
the 0.5 kb fragment is isolated from gel. The second PCR

fragment (35DE4/35F7) was digested with NdeI and HindIII and gel purified. These two fragments are then ligated into pBr/Ad35.PRn digested with NdeI and NotI to give pBr/Ad35.PR.TetOE4. The modification of the E4 promoter is

5   then transferred to the Ad35 helper cosmid clone by exchanging the PacI/SwaI fragment of the latter with the one from pBr/Ad35.PR.TetOE4 to give pWE/Ad35.pIX-rITR.TetOE4. pAAO-E-TATA.7xTetO was generated as follows. Two oligonucleotides were synthesized:

10   TATAplus: 5'-AGC TTT CTT ATA AAT TTT CAG TGT TAG ACT AGT AAA TTG CTT AAG-3' and
TATAmin: 5'-AGC TCT TAA GCA ATT TAC TAG TCT AAC ACT GAA AAT TTA TAA GAA-3'
(The underlined sequences form a modified TATA box).

15   The oligonucleotides were annealed to yield a double stranded DNA fragment with 5' overhangs that are compatible with HindIII digested DNA. The product of the annealing reaction was ligated into HindIII digested pGL3-Enhancer Vector (Promega) to yield pAAO-E-TATA. The clone that had

20   the HindIII site at the 5' end of the insert restored was selected for further cloning.
Next, the heptamerized tet-operator sequence was amplified from the plasmid pUHC-13-3 (Gossen and Bujard, 1992) in a PCR reaction using the Expand PCR system (Roche) according

25   to the manufacturers protocol. The following primers were used:
tet3: 5'- CCG GAG CTC CAT GGC CTA ACT CGA GTT TAC CAC TCC C-3'
tet5: 5'-CCC AAG CTT AGC TCG ACT TTC ACT TTT CTC-3'

30   The amplified fragment was digested with SstI and HindIII (these sites are present in tet3 and tet5 respectively) and cloned into SstI/HindIII digested pAAO-E-TATA giving rise to pAAO-E-TATA-7xtetO
To test the functionality of the generated pWE/Ad35.pIX-

35   rITR.TetOE4 cosmid clone, the DNA was digested with NotI. The left end of wtAd35 DNA was then amplified using primers

35F1 and 35R4 (see example 7). Following amplification, the
PCR mixture was purified and digested with SalI to remove
intact viral DNA. Then 4gr of both the digested
pWE/Ad35.pIX-rITR.TetOE4 and the PCR fragment was
5   cotransfected into PER.C6-tTA cells that were seeded in T25
flasks the day before. Transfected cells were transferred to
T80 flasks after two days and another two days later CPE was
obtained, showing that the cosmid backbone is functional.

10

Example 14
Generation of cell lines capable of complementing E1-deleted
Ad35 viruses

15   *Generation of pIG135 and pIG270*
Construct pIG.E1A.E1B contains E1 region sequences of Ad5
corresponding to nucleotides 459 to 3510 of the wt Ad5
sequence (Genbank accession number M72360) operatively
linked to the human phosphoglycerate kinase promoter (PGK)
20   and the Hepatitis B Virus polyA sequences. The generation of
this construct is described in WO97/00326. The E1 sequences
of Ad5 were replaced by corresponding sequences of Ad35 as
follows. pRSV.Ad35-E1 (described in example 8) was digested
with EcoRI and Sse8387I and the 3 kb fragment corresponding
25   to the Ad35 E1 sequences was isolated from gel. Construct
pIG.E1A.E1B was digested with Sse8387I completely and
partially with EcoRI. The 4.2 kb fragment corresponding to
vector sequences without the Ad5 E1 region but retaining the
PGK promoter were separated from other fragments on LMP
30   agarose gel and the correct band was excised from gel. Both
obtained fragments were ligated resulting in pIG.Ad35-E1.
This vector was further modified to remove the LacZ
sequences present in the pUC119 vector backbone. Hereto, the
vector was digested with BsaAI and BstXI and the large
35   fragment was isolated from gel. A double stranded oligo was
prepared by annealing the following two oligos:

BB1: 5'-GTG CCT AGG CCA CGG GG-3' and

BB2: 5'-GTG GCC TAG GCA C-3'

Ligation of the oligo and the vector fragment resulted in
construct pIG135. Correct insertion of the oligo restores
5   the BsaAI and BstXI sites and introduces a unique AvrII
site. Next, we introduced a unique site at the 3' end of the
Ad35-E1 expresion cassette in pIG135. Hereto, the construct
was digested with SapI and the 3' protruding ends were made
blunt by treatment with T4 DNA polymerase. The thus treated
10   linear plasmid was further digested with BsrGI and the large
vector containing fragment was isolated from gel. To restore
the 3' end of the HBVpolyA sequence and to introduce a
unique site, a PCR fragment was generated using the
following primers:
15   270F: 5'- CAC CTC TGC CTA ATC ATC TC -3' and
270R: 5'- GCT CTA GAA ATT CCA CTG CCT TCC ACC -3' .
The PCR was performed on pIG.Ad35.E1 DNA using Pwo
polymerase (Roche) according to the manufacturers
instructions. The obtained PCR product was digested with
20   BsrGI and dephosphorylated using Tsap enzym (LTI), the
latter to prevent insert dimerization on the BsrGI site. The
PCR fragment and the vector fragment were ligated to yield
construct pIG270.

25   *Ad35 E1 sequences are capable of transforming rat primary
cells*

New born WAG/RIJ rats were sacrificed at 1 week of gestation
and kidneys were isolated. After carefull removal of the
capsule, kidneys were disintegrated into a single cell
30   suspension by multiple rounds of incubation in trypsin/EDTA
(LTI) at 37 °C and collection of floating cells in cold PBS
containing 1% FBS. When most of the kidney was trypsinized
all cells were resuspended in DMEM supplemented with 10% FBS
and filtered through a sterile cheese cloth. Baby Rat Kidney
35   (BRK) cells obtained from one kidney were plated in 5 dishes
(Greiner, 6 cm). When a confluency of 70-80% was reached,

the cells were transfected with 1 or 5 μgr DNA/dish using
the CaPO₄ precipitation kit (LTI) according to the
manufacturers instructions. The following constructs were
used in separate transfections: pIG.E1A.E1B (expressing the
5 Ad5-E1 region), pRSV.Ad35-E1, pIG.Ad35-E1 and pIG270 (the
latter expressing the Ad35-E1). Cells were incubated at 37
°C, 5% CO₂ until foci of transformed cells appeared. Table
IV shows the number of foci that resulted from several
transfection experiments using circular or linear DNA. As
10 expected, the Ad5-E1 region efficiently transformed BRK
cells. Foci also appeared in the Ad35-E1 transfected cell
layer allthough with lower efficiency. The Ad35 transformed
foci appeared at a later time point: ˜2 weeks post
transfection compared with 7-10 days for Ad5-E1. These
15 experiments clearly show that the E1 genes of the B group
virus Ad35 are capable of transforming primary rodent cells.
This proves the functionality of the Ad35-E1 expression
constructs and confirms earlier findings of the transforming
capacity of the B-group viruses Ad3 and Ad7 (Dijkema, 1979).
20 To test whether the cells in the foci were really
transformed a few foci were picked and expanded. From the 7
picked foci at least 5 turned out to grow as established
cell lines.

25 *Generation of new packaging cells derived from primary human*
amniocytes

Amniotic fluid obtained after amnioscentesis was
centrifugated and cells were resuspended in AmnioMax medium
(LTI) and cultured in tissue culture flasks at 37 °C and 10
30 % CO₂. When cells were growing nicely (approximately one
cell division/24 hrs.), the medium was replaced with a 1:1
mixture of AmnioMax complete medium and DMEM low glucose
medium (LTI) supplemented with Glutamax I (end concentration
4mM, LTI) and glucose (end concentration 4.5 gr/L, LTI) and
35 10% FBS (LTI). For transfection ˜ 5x10⁵ cells were plated in
10 cm tissue culture dishes. The day after, cells were

transfected with 20 μgr of circular pIG270/dish using the
CaPO₄ transfection kit (LTI) according to manufacturers
instructions and cells were incubated overnight with the DNA
precipitate. The following day, cells were washed 4 times
5   with PBS to remove the precipitate and further incubated for
over three weeks until foci of transformed cells appeared.
Once a week the medium was replaced by fresh medium. Other
transfection agents like, but not limited to, LipofectAmine
(LTI) or PEI (Polyethylenimine, high molecular weight,
10 water-free, Aldrich) were used. Of these three agents PEI
reached the best transfection efficiency on primary human
amniocytes: ~1% blue cells 48 hrs. following transfection of
pAdApt35.LacZ.

Foci are isolated as follows. The medium is removed and
15 replaced by PBS after which foci are isolated by gently
scraping the cells using a 50-200 μl Gilson pipette with a
disposible filter tip. Cells contained in 10 μl PBS were
brought in a 96 well plate containing 15 μl trypsin/EDTA
(LTI) and a single cell suspension was obtained by pipetting
20 up and down and a short incubation at room temperature.
After addition of 200 μl of the above described 1:1 mixture
of AmnioMax complete medium and DMEM with supplements and
10% FBS, cells were further incubated. Clones that continued
to grow were expanded and analysed their ability to
25 complement growth of E1-deleted adenoviral vectors of
different sub-groups, specifically ones derived from B-
group viruses specifically from Ad35 or Ad11.

Generation of new packaging cell lines from human embryonic
30   *retinoblasts*

Human retina cells are isolated from the eyes of aborted
foetuses and cultured in DMEM medium (LTI) supplemented with
10% FBS (LTI). The day before transfection, ~5x10⁵ cells are
plated in 6 cm dishes and cultured overnight at 37 °C and
35 10% CO₂. Transfection is done using the CaPO₄ precipitation
kit (LTI) according to the manufacturers instructions. Each

dish is transfected with 8-10 μgr pIG270 DNA, either as a
circular plasmid or as a purified fragment. To obtain the
purified fragment, pIG270 was digested with AvrII and XbaI
and the 4 kb fragment corresponding to the Ad35 E1

5   expression cassette was isolated from gel by agarase
treatment (Roche). The following day, the precipitate is
washed away carefully by four washes with sterile PBS. Then
fresh medium is added and transfected cells are further
cultured untill foci of transformed cells appear. When large

10   enough (>100 cells) foci are picked and brought into 96-
wells as described above. Clones of transformed human
embryonic retinoblasts that continue to grow, are expanded
and tested for their ability to complement growth of E1-
deleted adenoviral vectors of different sub-groups

15   specifically ones derived from B-group viruses specifically
from Ad35 or Ad11.

New packaging cell lines derived from PER.C6
As described in example 8, it is possible to generate and

20   grow Ad35 E1-deleted viruses on PER.C6 cells with
cotransfection of an Ad35-E1 expression construct, e.g.
pRSV.Ad35.E1. However, large scale production of recombinant
adenoviruses using this method is cumbersome because for
each amplification step a transfection of the Ad35-E1

25   construct is needed. In addition, this method increases the
risk of non-homologous recombination between the plasmid and
the virus genome with high chances of generation of
recombinant viruses that incorporate E1 sequences resulting
in replication competent viruses. To avoid this, the

30   expression of Ad35-E1 proteins in PER.C6 has to be mediated
by integrated copies of the expression plasmid in the
genome. Since PER.C6 cells are already transformed and
express Ad5-E1 proteins, addition of extra Ad35-E1
expression may be toxic for the cells, however, it is not

35   impossible to stably transfect transformed cells with E1

89

proteins since Ad5-E1 expressing A549 cells have been
generated..

In an attempt to generate recombinant adenoviruses derived
from subgroup B virus Ad7, Abrahamsen et al. (1997) were not

5   able to generate E1-deleted viruses on 293 cells without
contamination of wt Ad7. Viruses that were picked after
plaque purification on 293-ORF6 cells (Brough et al., 1996)
were shown to have incorporated Ad7 E1B sequences by non-
homologous recombination. Thus, efficient propagation of Ad7

10   recombinant viruses proved possible only in the presence of
Ad7-E1B expression and Ad5-E4-ORF6 expression. The E1B
proteins are know to interact with cellular as well as viral
proteins (Bridge et al., 1993; White, 1995). Possibly, the
complex formed between the E1B 55K protein and E4-ORF6 which

15   is necessary to increase mRNA export of viral proteins and
to inhibit export of most cellular mRNAs, is critical and in
some way serotype specific. The above experiments suggest
that the E1A proteins of Ad5 are capable of complementing an
Ad7-E1A deletion and that Ad7-E1B expression in adenovirus

20   packaging cells on itself is not enough to generate a stable
complementing cell line. To test whether one or both of the
Ad35-E1B proteins is/are the limiting factor in efficient
Ad35 vector propagation on PER.C6 cells, we have generated
an Ad35 adapter plasmid that does contain the E1B promoter

25   and E1B sequences but lacks the promoter and the coding
region for E1A. Hereto, the left end of wtAd35 DNA was
amplified using the primers 35F1 and 35R4 (both described in
Example 7) with Pwo DNA polymerase (Roche) according to the
manufacturers' instructions. The 4.6 kb PCR product was

30   purified using the PCR purification kit (LTI) and digested
with SnaBI and ApaI enzymes. The resulting 4.2 kb fragment
was then purified from gel using the QIAExII kit (Qiagen).
Next, pAdApt35IP1 (Example 7) was digested with SnaBI and
ApaI and the 2.6 kb vector containing fragment was isolated

35   from gel using the GeneClean kit (BIO 101, Inc). Both

isolated fragments were ligated to give pBr/Ad35.leftITR-
pIX. Correct amplification during PCR was verified by a
functionality test as follows: The DNA was digested with
BstBI to liberate the Ad35 insert from vector sequences and
5   4 µgr of this DNA was cotransfected with 4 µgr of NotI
digested pWE/Ad35.pIX-rITR (Example 7) into PER.C6 cells.
The transfected cells were passaged to T80 flasks at day 2
and again two days later CPE had formed showing that the new
pBr/Ad35.leftITR-pIX construct contains functional E1
10   sequences. The pBr/Ad35.leftITR-pIX construct was then
further modified as follows. The DNA was digested with SnaBI
and HindIII and the 5' HindII overhang was filled in using
Klenow enzyme. Religation of the digested DNA and
transformation into competent cells (LTI) gave construct
15   pBr/Ad35leftITR-pIXΔE1A. This latter constuct contains the
left end 4.6 kb of Ad35 except for E1A sequences between bp
450 and 1341 (numbering according to wtAd35, Figure 6) and
thus lacks the E1A promoter and most of the E1A coding
sequences. pBr/Ad35.leftITR-pIXΔE1A was then digested with
20   BstBI and 2 µgr of this construct was cotransfected with 6 µ
gr of NotI digested pWE/Ad35.pIX-rITR (Example 7) into
PER.C6 cells. One week following transfection full CPE had
formed in the transfected flasks.
This experiment shows that the Ad35-E1A proteins are
25   functionally complemented by Ad5-e1A expression in PER.C6
cellsand that at least one of the Ad35-E1B proteins cannot
be complemented by Ad5-E1 expression in PER.C6. It further
shows that it is possible to make a complementing cell line
for Ad35 E1-deleted viruses by expressing Ad35-E1B proteins
30   in PER.C6. Stable expression of Ad35-E1B sequences from
integrated copies in the genome of PER.C6 cells may be
driven by the E1B promoter and terminated by a heterologous
poly-adenylation signal like, but not limited to, the HBVpA.
The heterologous pA signal is necessary to avoid overlap
35   between the E1B insert and the recombinant vector, since the
natural E1B termination is located in the pIX transcription

unit which has to be present on the adenoviral vector.
Alternatively, the E1B sequences may be driven by a
heterologous promoter like, but not limited to the human PGK
promoteror by an inducible promoter like, but not limited to
5   the 7xtetO promoter (Gossen and Bujard, 1992). Also in these
cases the transcription termination is mediated by a
heterologous pA sequence, e.g. the HBV pA. The Ad35-E1B
sequences at least comprise one of the coding regions of the
E1B 21K and the E1B 55K proteins located between nucleotides
10   1611 and 3400 of the wt Ad35 sequence. The insert may also
include (part of the) Ad35-E1B sequences between nucleotides
1550 and 1611 of the wt Ad35 sequence.

                              Example 15

15   Generation of producer cell lines for the production of
recombinant adenoviral vectors deleted in early region 1 and
early region 2A

Generation of PER.C6-tTA cells
20   Here is described the generation of cell lines for the
production of recombinant adenoviral vectors that are
deleted in early region 1 (E1) and early region 2A (E2A).
The producer cell lines complement for the E1 and E2A
deletion from recombinant adenoviral vectors *in trans* by
25   constitutive expression of both E1 and E2A genes. The pre-
established Ad5-E1 transformed human embryo retinoblast cell
line PER.C6 (WO 97/00326) was further equipped with E2A
expression cassettes.
          The adenoviral E2A gene encodes a 72 kDa DNA Binding
30   Protein with has a high affinity for single stranded DNA.
Because of its function, constitutive expression of DBP is
toxic for cells. The ts125E2A mutant encodes a DBP which has
a Pro→Ser substitution of amino acid 413. Due to this
mutation, the ts125E2A encoded DBP is fully active at the
35   permissive temperature of 32°C, but does not bind to ssDNA
at the non-permissive temperature of 39°C. This allows the

generation of cell lines that constitutively express E2A, which is not functional and is not toxic at the non-permissive temperature of 39°C. Temperature sensitive E2A gradually becomes functional upon temperature decrease and

5   becomes fully functional at a temperature of 32°C, the permissive temperature.

A. Generation of plasmids expressing the wild type E2A- or temperature sensitive ts125E2A gene.

10   pcDNA3wtE2A: The complete wild-type early region 2A (E2A) coding region was amplified from the plasmid pBR/Ad.Bam-rITR (ECACC deposit P97082122) with the primers DBPpcr1 and DBPpcr2 using the Expand™ Long Template PCR system according to the standard protocol of the supplier

15   (Boehringer Mannheim). The PCR was performed on a Biometra Trio Thermoblock, using the following amplification program: 94°C for 2 minutes, 1 cycle; 94°C for 10 seconds + 51°C for 30 seconds + 68°C for 2 minutes, 1 cycle; 94°C for 10 seconds + 58°C for 30 seconds + 68°C for 2 minutes, 10

20   cycles; 94°C for 10 seconds + 58°C for 30 seconds + 68°C for 2 minutes with 10 seconds extension per cycle, 20 cycles; 68°C for 5 minutes, 1 cycle. The primer DBPpcr1: CG*G GAT CC*G *CCA CCA TGG CCA GTC GGG AAG AGG AG* (5' to 3') contains a unique *Bam*HI restriction site (underlined) 5' of the Kozak

25   sequence (italic) and start codon of the E2A coding sequence. The primer DBPpcr2: CG*G AAT TCT* **TAA AAA TCA AAG GGG TTC TGC CGC** (5' to 3') contains a unique *Eco*RI restriction site (underlined) 3' of the stop codon of the E2A coding sequence. The bold characters refer to sequences

30   derived from the E2A coding region. The PCR fragment was digested with *Bam*HI/*Eco*RI and cloned into *Bam*HI/*Eco*RI digested pcDNA3 (Invitrogen), giving rise to pcDNA3wtE2A.

pcDNA3tsE2A: The complete ts125E2A-coding region was amplified from DNA isolated from the temperature sensitive

35   adenovirus mutant H5ts125. The PCR amplification procedure

was identical to that for the amplification of wtE2A. The
PCR fragment was digested with *BamHI/EcoRI* and cloned into
BamHI/EcoRI digested pcDNA3 (Invitrogen), giving rise to
pcDNA3tsE2A. The integrity of the coding sequence of wtE2A

5   and tsE2A was confirmed by sequencing.

B. Growth characteristics of producer cells for the
production of recombinant adenoviral vectors cultured at 32-
, 37- and 39°C.

10   PER.C6 cells were cultured in Dulbecco's Modified Eagle
Medium (DMEM, Gibco BRL) supplemented with 10% Fetal Bovine
Serum (FBS, Gibco BRL) and 10mM $MgCl_2$ in a 10% CO_2
atmosphere at either 32°C, 37°C or 39°C. At day 0, a total of
1×10^6 PER.C6 cells were seeded per $25cm^2$ tissue culture

15   flask (Nunc) and the cells were cultured at either 32°C,
37°C or 39°C. At day 1-8, cells were counted. Figure 30
shows that the growth rate and the final cell density of the
PER.C6 culture at 39°C are comparable to that at 37°C. The
growth rate and final density of the PER.C6 culture at 32°C

20   were slightly reduced as compared to that at 37°C or 39°C.
No significant cell death was observed at any of the
incubation temperatures. Thus PER.C6 performs very well both
at 32°C and 39°C, the permissive and non-permissive
temperature for ts125E2A, respectively.

25

C. Transfection of PER.C6 with E2A expression vectors;
colony formation and generation of cell lines
One day prior to transfection, 2×10^6 PER.C6 cells were
seeded per 6 cm tissue culture dish (Greiner) in DMEM,

30   supplemented with 10% FBS and 10mM $MgCl_2$ and incubated at
37°C in a 10% CO_2 atmosphere. The next day, the cells were
transfected with 3, 5 or 8μg of either pcDNA3, pcDNA3wtE2A
or pcDNA3tsE2A plasmid DNA per dish, using the LipofectAMINE
PLUS™ Reagent Kit according to the standard protocol of the

35   supplier (Gibco BRL), except that the cells were transfected
at 39°C in a 10% CO_2 atmosphere. After the transfection, the

cells were constantly kept at 39°C, the non-permissive temperature for ts125E2A. Three days later, the cells were put in DMEM supplemented with 10% FBS, 10mM MgCl₂ and 0.25mg/ml G418 (Gibco BRL), and the first G418 resistant

5   colonies appeared at 10 days post transfection. As shown in table 1, there was a dramatic difference between the total number of colonies obtained after transfection of pcDNA3 (˜200 colonies) or pcDNA3tsE2A (˜100 colonies) and pcDNA3wtE2A (only 4 colonies). These results indicate that

10 the toxicity of constitutively expressed E2A can be overcome by using a temperature sensitive mutant of E2A (ts125E2A) and culturing of the cells at the non-permissive temperature of 39°C.

From each transfection, a number of colonies was picked

15 by scraping the cells from the dish with a pipette. The detached cells were subsequently put into 24 wells tissue culture dishes (Greiner) and cultured further at 39°C in a 10% CO₂ atmosphere in DMEM, supplemented with 10% FBS, 10mM MgCl₂ and 0.25mg/ml G418. As shown in table 1, 100% of the

20 pcDNA3 transfected colonies (4/4) and 82% of the pcDNA3tsE2A transfected colonies (37/45) were established to stable cell lines (the remaining 8 pcDNA3tsE2A transfected colonies grew slowly and were discarded). In contrast, only 1 pcDNA3wtE2A-transfected colony could be established. The

25 other 3 died directly after picking.

Next, the E2A expression levels in the different cell lines were determined by Western blotting. The cell lines were seeded on 6 well tissue culture dishes and sub-confluent cultures were washed twice with PBS (NPBI) and

30 lysed and scraped in RIPA (1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS in PBS, supplemented with 1mM phenylmethylsulfonylfluoride and 0.1 mg/ml trypsin inhibitor). After 15 minutes incubation on ice, the lysates were cleared by centrifugation. Protein concentrations were

35 determined by the Bio-Rad protein assay, according to standard procedures of the supplier (BioRad). Equal amounts

of whole-cell extract were fractionated by SDS-PAGE on 10%
gels. Proteins were transferred onto Immobilon-P membranes
(Millipore) and incubated with the αDBP monoclonal antibody
B6. The secondary antibody was a horseradish-peroxidase-
5   conjugated goat anti mouse antibody (BioRad). The Western
blotting procedure and incubations were performed according
to the protocol provided by Millipore. The complexes were
visualized with the ECL detection system according to the
manufacturer's protocol (Amersham). Figure 31 shows that all
10 of the cell lines derived from the pcDNA3tsE2A transfection
expressed the 72-kDa E2A protein (left panel, lanes 4-14;
middle panel, lanes 1-13; right panel, lanes 1-12). In
contrast, the only cell line derived from the pcDNAwtE2A
transfection did not express the E2A protein (left panel,
15 lane 2). No E2A protein was detected in extract from a cell
line derived from the pcDNA3 transfection (left panel, lane
1), which served as a negative control. Extract from PER.C6
cells transiently transfected with pcDNA3ts125 (left panel,
lane 3) served as a positive control for the Western blot
20 procedure. These data confirmed that constitutive expression
of wtE2A is toxic for cells and that using the ts125 mutant
of E2A could circumvent this toxicity.

D. Complementation of E2A deletion in adenoviral vectors on
25 PER.C6 cells constitutively expressing full-length ts125E2A.
The adenovirus Ad5.dl802 is an Ad 5 derived vector deleted
for the major part of the E2A coding region and does not
produce functional DBP. Ad5.dl802 was used to test the E2A
trans-complementing activity of PER.C6 cells constitutively
30 expressing ts125E2A. Parental PER.C6 cells or PER.C6tsE2A
clone 3-9 were cultured in DMEM, supplemented with 10% FBS
and 10mM $MgCl_2$ at 39°C and 10% CO_2 in 25 cm^2 flasks and
either mock infected or infected with Ad5.dl802 at an m.o.i.
of 5. Subsequently the infected cells were cultured at 32°C
35 and cells were screened for the appearance of a cytopathic
effect (CPE) as determined by changes in cell morphology and

detachment of the cells from the flask. Full CPE appeared in the Ad5.dl802 infected PER.C6tsE2A clone 3-9 within 2 days. No CPE appeared in the Ad5.dl802 infected PER.C6 cells or the mock infected cells. These data showed that PER.C6 cells

5   constitutively expressing ts125E2A complemented in trans for the E2A deletion in the Ad5.dl802 vector at the permissive temperature of 32°C.

       E. Serum-free suspension culture of PER.C6tsE2A cell lines.

10   Large-scale production of recombinant adenoviral vectors for human gene therapy requires an easy and scaleable culturing method for the producer cell line, preferably a suspension culture in medium devoid of any human or animal constituents. To that end, the cell line PER.C6tsE2A c5-9

15   (designated c5-9) was cultured at 39°C and 10% CO_2 in a 175 cm^2 tissue culture flask (Nunc) in DMEM, supplemented with 10% FBS and 10mM $MgCl_2$. At sub-contluency (70-80% confluent), the cells were washed with PBS (NPBI) and the medium was replaced by 25 ml serum free suspension medium

20   Ex-cell™ 525 (JRH) supplemented with 1 x L-Glutamine (Gibco BRL), hereafter designated SFM. Two days later, cells were detached from the flask by flicking and the cells were centrifuged at 1,000 rpm for 5 minutes. The cell pellet was resuspended in 5 ml SFM and 0.5 ml cell suspension was

25   transferred to a 80 cm^2 tissue culture flask (Nunc), together with 12 ml fresh SFM. After 2 days, cells were harvested (all cells are in suspension) and counted in a Burker cell counter. Next, cells were seeded in a 125 ml tissue culture erlenmeyer (Corning) at a seeding density of

30   3×10^5 cells per ml in a total volume of 20 ml SFM. Cells were further cultured at 125 RPM on an orbital shaker (GFL) at 39°C in a 10% CO_2 atmosphere. Cells were counted at day 1-6 in a Burker cell counter. In Figure 4, the mean growth curve from 8 cultures is shown. PER.C6tsE2A c5-9 performed

35   well in serum free suspension culture. The maximum cell

density of approximately 2×10^6 cells per ml is reached within 5 days of culture.

F. Growth characteristics of PER.C6 and PER.C6/E2A at 37°C
5   and 39°C.
PER.C6 cells or PER.C6ts125E2A (c8-4) cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco BRL) supplemented with 10% Fetal Bovine Serum (FBS, Gibco BRL) and 10mM $MgCl_2$ in a 10% CO_2 atmosphere at either 37°C
10   (PER.C6) or 39°C (PER.C6ts125E2A c8-4). At day 0, a total of 1×10^6 cells were seeded per $25cm^2$ tissue culture flask (Nunc) and the cells were cultured at the respective temperatures. At the indicated time points, cells were counted. The growth of PER.C6 cells at 37°C was comparable
15   to the growth of PER.C6ts125E2A c8-4 at 39°C (Figure 33). This shows that constitutive expression of ts125E2A encoded DBP had no adverse effect on the growth of cells at the non-permissive temperature of 39°C.

20   G. Stability of PER.C6ts125E2A
For several passages, the PER.C6ts125E2A cell line clone 8-4 was cultured at 39°C and 10% CO_2 in a 25 cm^2 tissue culture flask (Nunc) in DMEM, supplemented with 10% FBS and 10 mM $MgCl_2$ in the absence of selection pressure (G418). At sub-
25   confluency (70-80% confluent), the cells were washed with PBS (NPBI) and lysed and scraped in RIPA (1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS in PBS, supplemented with 1mM phenylmethylsulfonylfluoride and 0.1 mg/ml trypsin inhibitor). After 15 minutes incubation on ice, the lysates
30   were cleared by centrifugation. Protein concentrations were determined by the BioRad protein assay, according to standard procedures of the supplier (BioRad). Equal amounts of whole-cell extract were fractionated by SDS-PAGE in 10% gels. Proteins were transferred onto Immobilon-P membranes
35   (Millipore) and incubated with the αDBP monoclonal antibody B6. The secondary antibody was a horseradish-peroxidase-

conjugated goat anti mouse antibody (BioRad). The Western
blotting procedure and incubations were performed according
to the protocol provided by Millipore. The complexes were
visualized with the ECL detection system according to the
5   manufacturer's protocol (Amersham). The expression of
ts125E2A encoded DBP was stable for at least 16 passages,
which is equivalent to approximately 40 cell doublings
(Figure 34). No decrease in DBP levels was observed during
this culture period, indicating that the expression of
10 ts125E2A was stable, even in the absence of G418 selection
pressure.

Example 16
    Generation of tTA expressing packaging cell lines

15

*A. Generation of a plasmid from which the tTA gene is
expressed.*

pcDNA3.1-tTA: The tTA gene, a fusion of the tetR and VP16
genes, was removed from the plasmid pUHD 15-1 (Gossen and
20 Bujard, 1992) by digestion using the restriction enzymes
BamHI and *EcoRI*. First, pUHD15-1 was digested with *EcoRI*.
The linearized plasmid was treated with Klenow enzyme in the
presence of dNTPs to fill in the *EcoRI* sticky ends. Then,
the plasmid was digested with *BamHI*. The resulting fragment,
25 1025 bp in length, was purified from agarose. Subsequently,
the fragment was used in a ligation reaction with
BamHI/EcoRV digested pcDNA 3.1 HYGRO (-) (Invitrogen) giving
rise to pcDNA3.1-tTA. After transformation into competent *E.
Coli* DH5α (Life Techn.) and analysis of ampiciline resistant
30 colonies, one clone was selected that showed a digestion
pattern as expected for pcDNA3.1-tTA.

B. Transfection of PER.C6 and PER.C6/E2A with the tTA
expression vector; colony formation and generation of cell
lines

5   One day prior to transfection, 2×10^6 PER.C6 or
PER.C6/E2A cells were seeded per 60 mm tissue culture dish
(Greiner) in Dulbecco's modified essential medium (DMEM,
Gibco BRL) supplemented with 10% FBS (JRH) and 10 mM $MgCl_2$
and incubated at 37°C in a 10% CO_2 atmosphere. The next day,
10   cells were transfected with 4-8 μg of pcDNA3.1-tTA plasmid
DNA using the LipofectAMINE PLUS™ Reagent Kit according to
the standard protocol of the supplier (Gibco BRL). The cells
were incubated with the LipofectAMINE PLUS™-DNA mixture for
four hours at 37°C and 10% CO_2. Then, 2 ml of DMEM
15   supplemented with 20% FBS and 10 mM $MgCl_2$ was added and
cells were further incubated at 37°C and 10% CO_2. The next
day, cells were washed with PBS and incubated in fresh DMEM
supplemented with 10% FBS, 10 mM $MgCl_2$ at either 37°C
(PER.C6) or 39°C (Per.C6/E2A) in a 10% CO_2 atmosphere for
20   three days. Then, the media were exchanged for selection
media; PER.C6 cells were incubated with DMEM supplemented
with 10% FBS, 10 mM $MgCl_2$ and 50 μg/ml hygromycin B (GIBCO)
while PER.C6/E2A cells were maintained in DMEM supplemented
with 10% FBS, 10 mM $MgCl_2$ and 100 μg/ml hygromycin B.
25   Colonies of cells that resisted the selection appeared
within three weeks while nonresistant cells died during this
period.

       From each transfection, a number of independent,
hygromycin resistant cell colonies were picked by scraping
30   the cells from the dish with a pipette and put into 2.5 cm^2
dishes (Greiner) for further growth in DMEM containing 10%
FBS, 10 mM $MgCl_2$ and supplemented with 50 μg/ml (PERC.6
cells) or 100 μg/ml (PERC.6/E2A cells) hygromycin in a 10%
CO_2 atmosphere and at 37°C or 39°C, respectively.

Next, it was determined whether these hygromycin-
resistant cell colonies expressed functional tTA protein.
Therefore, cultures of PER.C6/tTA or PER/E2A/tTA cells were
transfected with the plasmid pUHC 13-3 that contains the
5   reporter gene luciferase under the control of the 7xtetO
promoter (Gossens and Bujard, 1992). To demonstrate that the
expression of luciferase was mediated by tTA, one half of
the cultures was maintained in medium without doxycycline.
The other half was maintained in medium with 8 µg/ml
10   doxycycline (Sigma). The latter drug is an analogue of
tetracycline and binds to tTA and inhibits its activity. All
PER.C6/tTA and PER/E2A/tTA cell lines yielded high levels of
luciferase, indicating that all cell lines expressed the tTA
protein (Figure 35). In addition, the expression of
15   luciferase was greatly suppressed when the cells were
treated with doxycycline. Collectively, the data showed that
the isolated and established hygromycin-resistant PER.C6 and
PER/E2A cell clones all expressed functional tTA.

Legend to the figures:

Figure 1:

5   Bar graph showing the percentage of serum samples positive
     for neutralisation for each human wt adenovirus tested (see
     example1 for description of the neutralisation assay).

Figure 2:

10   Graph showing absence of correlation between the VP/CCID50
     ratio and the percentage of neutralisation.

Figure 3:
     Schematic representation of a partial restriction map of

15   Ad35 (taken from Kang et al., 1989) and the clones generated
     to make recombinant Ad35-based viruses.

Figure 4: Bar graph presenting the percentage sera samples
     that show neutralizing activity to a selection of adenovirus

20   serotypes. Sera were derived from healthy volunteers from
     Belgium and the UK.

Figure 5: Bar graph presenting the percentage sera samples
     that show neutralizing activity to adenovirus serotypes 5,

25   11, 26, 34, 35, 48 and 49. Sera were derived from five
     different locations in Europe and the United States.

Figure 6: Sequence of human adenovirus type 35. As explained
     in the text the nucleotide sequence of the terminal ends of

30   the virus are not definite resolved.
     Figure 7: Map of pAdApt
     Figure 8: Map of pIPspAdapt
     Figure 9: Map of pIPspAdapt1
     Figure 10: Map of pIPspAdapt3

35   Figure 11: Map of pAdApt35IP3

Figure 12: Map of pAdApt35IP1

Figure 13: Schematic representation of the steps undertaken
to construct pWE.Ad35.pIX-rITR

Figure 14: Map of pWE.Ad35.pIX-rITR

5   Figure 15: Map of pRSV.Ad35-E1

Figure 16: Map of PGKneopA

Figure 17: Map of pRSVpNeo

Figure 18: Map of pRSVhbvNeo

10   Figure 19: Flow cytometric analyses on Green fluorescent
protein (GFP) expression in human TF-1 cells. Non-transduced
TF-1 cells were used to set a background level of 1%. GFP
expression in cells transduced with Ad5, Ad5.Fib16,
Ad5.Fib17, Ad5.Fib40-L, Ad5.Fib35, and Ad5.Fib51 is shown.

15 .

Figure 20: Transduction of primary human fibroblast-like
stroma. Cells were analyzed 48 hours after a two hour
exposure to the different chimaeric fiber viruses. Shown is
percentage of cells found positive for the transgene: green

20   fluorescent protein (GFP) using a flow cytometer. Non-
transduced stroma cells were used to set a background at 1%.
Results of different experiments (n=3) are shown ± standard
deviation.

25   Figure 21: Transduction of primary human fibroblast-like
stroma, CD34$^+$ cells and CD34$^+$Lin$^-$ cells. Cells were analyzed
5 days after a two hour exposure to the different chimaeric
fiber viruses. Shown is percentage of cells found positive
for the transgene: green fluorescent protein (GFP) using a

30   flow cytometer. Non-transduced cells were used to set a
background at 1%. Also shown is the number of GFP positive .
events divided by the total number of events analysed
(between brackets).

35   Figure 22 A) Flow cytometric analysis of GFP positive cells
after transduction of CD34$^+$ cells with Ad5.Fib51. All cells

gated in R2-R7 are positive for CD34 but differ in their
expression of early differentiation markers CD33, CD38, and
CD71 (Lin). Cells in R2 are negative for CD333, CD38, CD71
whereas cells in R7 are positive for hese markers. To

5   demonstrate specificity of Ad5.Fib51 the percentage of GFP
positive cells was determined in R2-R7 which proofed to
decline from 91% (R2) to 15% (R7). B) Identical experiment
as shown under A (X-axes is R2-R7) but for the other Ad
fiber chimaeric viruses showing that Ad5.Fib35, and

10   Ad5.Fib16 behave similar as Ad5.Fib51.

Figure 23: Alignment of the chimeric fiber proteins of
Ad5fib16, Ad5fib35 and Ad5fib51 with the Ad5 fiber sequence.

15   Figure 24: Toxicity of Adenovirus exposure to primitive
human Bone marrow cells and Stem cells. Cell cultures were
counted just before before and 5 days after adenovirus
transduction. Shown is the percentage of primitive human
bone marrow cells (CD34$^+$) and HSCs (CD34$^+$Lin$^-$) recovered as

20   compared to day 0.

Figure 25: Transduction of immature DCs at a virus dose of
100 or 1000 virus particles per cell. Virus tested is Ad5
and Ad5 based vectors carrying the fiber of serotype 12

25   (Ad5.Fib12), 16 (Ad5.Fib16), 28 (Ad5.Fib28), 32 (Ad5.Fib32),
the long fiber of 40 (Ad5.Fib40-L, 49 (Ad5.Fib49), 51
(Ad5.Fib51).Luciferase transgene expression is expressed as
relative light units per microgram of protein.

30   Figure 26: Flow cytometric analyses of LacZ expression on
immature and mature DCs transduced with 10000 virus
particles per cell of Ad5 or the fiber chimaeric vectors
Ad5.Fib16, Ad5.Fib40-L, or Ad5.Fib51. Percentages of cells
scored positive are shown in upper left corner of each

35   histogram.

Figure 27: Luciferase transgene expression in human immature DCs measured 48 hours after transduction with 1000 or 5000 virus particles per cell. Virus tested were fiber chimaeric viruses carrying the fiber of subgroup B members (serotypes

5   11, 16, 35, and 51).

Figure 28: Green fluorescent protein (GFP) expression in immature human DCs48 hours after transduction with 1000 virus particles per cell of Ad5, Ad5.Fib16, and Ad5.Fib35.

10   Non-transduced cells were used to set a background level of approximately 1% (-).

Figure 29: Transduction of mouse and chimpanzee DCs. Luciferase transgene expression measured in mouse DCs 48

15   hours after transduction is expressed as relative light units per microgram of protein.Chimpanzee DCs were measured 48 hours after transduction usinf a flow cycometer. GFP expression demonstrates the poor transduction of Ad (35) in contrast to Ad5.Fib35 (66%).

20

Figure 30: Temperature dependent growth of PER.C6. PER.C6 cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% Fetal Bovine Serum (FBS, Gibco BRL) and 10mM $MgCl_2$ in a 10% CO_2 atmosphere at either 32°C,

25   37°C or 39°C. At day 0, a total of 1×10^6 PER.C6 cells were seeded per $25cm^2$ tissue culture flask (Nunc) and the cells were cultured at either 32°C, 37°C or 39°C. At day 1-8, cells were counted. The growth rate and the final cell density of the PER.C6 culture at 39°C are comparable to that at 37°C.

30   The growth rate and final density of the PER.C6 culture at 32°C were slightly reduced as compared to that at 37°C or 39°C.

PER.C6 cells were seeded at a density of 1×10^6 cells per

25 cm^2 tissue culture flask and cultured at either 32-, 37-

35   or 39°C. At the indicated time points, cells were counted in

a Burker cell counter. PER.C6 grows well at both 32-, 37-
and 39^{0}C.

Figure 31: DBP levels in PER.C6 cells transfected with
5   pcDNA3, pcDNA3wtE2A or pcDNA3ts125E2A.
    Equal amounts of whole-cell extract were fractionated by
    SDS-PAGE on 10% gels. Proteins were transferred onto
    Immobilon-P membranes and DBP protein was visualized using
    the αDBP monoclonal B6 in an ECL detection system. All of
10 the cell lines derived from the pcDNA3ts125E2A transfection
    express the 72-kDa E2A-encoded DBP protein (left panel,
    lanes 4-14; middle panel, lanes 1-13; right panel, lanes 1-
    12). In contrast, the only cell line derived from the
    pcDNAwtE2A transfection did not express the DBP protein
15 (left panel, lane 2). No DBP protein was detected in extract
    from a cell line derived from the pcDNA3 transfection (left
    panel, lane 1), which serves as a negative control. Extract
    from PER.C6 cells transiently transfected with pcDNA3ts125
    (left panel, lane 3) served as a positive control for the
20 Western blot procedure. These data confirm that constitutive
    expression of wtE2A is toxic for cells and that using the
    ts125 mutant of E2A can circumvent this toxicity.

    Figure 32: Suspension growth of PER.C6ts125E2A C5-9.
25 The tsE2A expressing cell line PER.C6tsE2A.c5-9 was cultured
    in suspension in serum free Ex-cell™. At the indicated time
    points, cells were counted in a Burker cell counter. The
    results of 8 independent cultures are indicated. PER.C6tsE2A
    grows well in suspension in serum free Ex-cell™ medium.
30
    Figure 33: Growth curve PER.C6 and PER.C6tsE2A.
    PER.C6 cells or PER.C6ts125E2A (c8-4) cells were cultured at
    37^{0}C or 39^{0}C, respectively. At day 0, a total of 1 x 10^6
    cells was seeded per 25cm^2 tissue culture flask. At the
35 indicated time points, cells were counted. The growth of

PER.C6 cells at 37°C is comparable to the growth of
PER.C6ts125E2A c8-4 at 39°C. This shows that constitutive
overexpression of ts125E2A has no adverse effect on the
growth of cells at the non-permissive temperature of 39°C.

5

Figure 34: Stability of PER.C6ts125E2A.
For several passages, the PER.C6ts125E2A cell line clone 8-4
was cultured at 39°C in medium without G418. Equal amounts
of whole-cell extract from different passage numbers were
10 fractionated by SDS-PAGE on 10% gels. Proteins were
transferred onto Immobilon-P membranes and DBP protein was
visualized using the αDBP monoclonal B6 in an ECL detection
system. The expression of ts125E2A encoded DBP is stable for
at least 16 passages, which is equivalent to approximately
15 40 cell doublings. No decrease in DBP levels were observed
during this culture period, indicating that the expression
of ts125E2A is stable, even in the absence of G418 selection
pressure.

20 Figure 35: tTA activity in hygromycin resistent PER.C6/tTA
(A) and PER/E2A/tTA (B) cells.
Sixteen independent hygromycin resistent PER.C6/tTA cell
colonies and 23 independent hygromycin resistent PER/E2A/tTA
cell colonies were grown in 10 cm² wells to sub-confluency
25 and transfected with 2 μg of pUHC 13-3 (a plasmid that
contains the reporter gene luciferase under the control of
the 7xtetO promoter). One half of the cultures was
maintained in medium containing doxycycline to inhibit the
activity of tTA. Cells were harvested at 48 hours after
30 transfection and luciferase activity was measured. The
luciferase activity is indicated in relative light units
(RLU) per μg protein.

Table I:

Serotype	Elution [NaCl] mM	VP/ml	CCID50	$\log_{10}$ VP/CCID50 ratio
1	597	8.66×10^{10}	5.00×10^7	3.2
2	574	1.04×10^{12}	3.66×10^{11}	0.4
3	131	1.19×10^{11}	1.28×10^7	4.0
4	260	4.84×10^{11}	2.50×10^8	3.3
5	533	5.40×10^{11}	1.12×10^{10}	1.7
6	477	1.05×10^{12}	2.14×10^{10}	1.7
7	328	1.68×10^{12}	2.73×10^9	2.4
9	379	4.99×10^{11}	3.75×10^7	4.1
10	387	8.32×10^{12}	1.12×10^9	3.9
12	305	3.64×10^{11}	1.46×10^7	4.4
13	231	4.37×10^{12}	7.31×10^8	3.8
15	443	5.33×10^{12}	1.25×10^9	3.6
16	312	1.75×10^{12}	5.59×10^8	3.5
17	478	1.39×10^{12}	1.45×10^9	3.0
19	430	8.44×10^{11}	8.55×10^7	4.0
20	156	1.41×10^{11}	1.68×10^7	3.9
21	437	3.21×10^{11}	1.12×10^8	3.5
22	365	$1.43 \times 10^{\cdot}$	5.59×10	3.4
23	132	2.33×10^{11}	1.57×10^7	4.2
24	405	5.12×10^{12}	4.27×10^8	4.1
25	405	7.24×10^{11}	5.59×10^7	4.1
26	356	1.13×10^{12}	1.12×10^8	4.0
27	342	2.00×10^{12}	1.28×10^8	4.2
28	347	2.77×10^{12}	5.00×10^7	4.7
29	386	2.78×10^{11}	2.00×10^7	4.1
30	409	1.33×10^{12}	5.59×10^8	3.4
31	303	8.48×10^{10}	2.19×10^7	3.6
33	302	1.02×10^{12}	1.12×10^7	5.0
34	425	1.08×10^{12}	1.63×10^{11}	0.8
35	446	3.26×10^{12}	1.25×10^{11}	1.4
36	325	9.26×10^{12}	3.62×10^9	3.4
37	257	5.86×10^{12}	2.8×10^9	3.3
38	337	3.61×10^{12}	5.59×10^7	4.8
39	241	3.34×10^{11}	1.17×10^7	4.5
42	370	1.95×10^{12}	1.12×10^8	4.2

Continued on next page.

Serotype #	Elution [NaCl] mM	VP/ml	CCID50	$\log_{10}$ VP/CCID50 ratio
43	284	2.42×10^{12}	1.81×10^{8}	4.1
44	295	8.45×10^{11}	2.00×10^{7}	4.6
45	283	5.20×10^{11}	2.99×10^{7}	4.2
46	282	9.73×10^{12}	2.50×10^{8}	4.6
47	271	5.69×10^{11}	3.42×10^{7}	4.2
48	264	1.68×10^{12}	9.56×10^{8}	3.3
49	332	2.20×10^{12}	8.55×10^{7}	4.4
50	459	7.38×10^{12}	2.80×10^{9}	3.4
51	450	8.41×10^{11}	1.88×10^{8}	3.7

Legend to table I:

All human adenoviruses used in the neutralisation experiments

5   were produced on PER.C6 cells (ECACC deposit number 96022940)

(Fallaux et al., 1998) and purified on CsCl as described in

example 1. The NaCl concentration at which the different

serotypes eluted from the HPLC column is shown. Virus

particles/ml (VP/ml) were calculated from an Ad5 standard.

10   The titer in the experiment (CCID50) was determined on PER.C6

cells (ECACC deposit number 96022940) as described in example

1 by titrations performed in parallel with the neutralisation

experiment. The CCID50 is shown for the 44 viruses used in

this study and reflects the dilution of the virus needed to

15   obtain CPE in 50% of the wells after 5 days. The ratio of

VP/CCID50 is depicted in $\log_{10}$ and is a measurement of the

infectivity of the different batches on PER.C6 cells (ECACC

deposit number 96022940).

Table II.　AdApt35.LacZ viruses escape neutralization by

human serum.

Virus	Human serum dilution					
	no serum	10x	50x	250x	1250x	6250x
AdApt5.LacZ moi: 5 VP/cell	100 %	0 %	0 %	1 %	40 %	80 %
AdApt35.LacZ 250 μl crude lysate	100 %	100 %	100 %	100 %	100 %	100 %

5

Table III: Percentage of synovial fluid samples containing

neutralizing activity (NA) to wt adenoviruses of different

serotypes.

10

	% of SF samples with NA (all positives)	% of SF samples with NA (positives at ≥64x dilution)
Ad5	72	59
Ad26	66	34
Ad34	45	19
Ad35	4	0
Ad48	42	4

Table IV: The numbers of foci obtained with the different E1

expression constructs in BRK transformation experiments.

5   Average # of foci/dish:

	Construct	1 μgr	5 μgr
Experiment 1	pIG.E1A.E1 B	nd	60
	pIG.E1A.E1 B	nd	35
	pRSVAd35E1	0	3
	pIG.Ad35.E 1	3	7
Experiment 2	pIG.E1A.E1 B	37	nd
	pIG.Ad35.E 1	nd	2
Experiment 3	pIG.E1A.E1 B	nd	140
	pIG.Ad35.E 1	nd	20
	pIG270	nd	30

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CLAIMS

1.   A gene delivery vehicle comprising at least one of the adenovirus serotype 35 elements or a functional equivalent thereof, responsible for avoiding or deminishing neutralising activity against adenoviral elements by the host to which the
5   gene is to be delivered and a gene of interest.

2.   Adenovirus serotype 35 or a functional homologue thereof or a chimaeric virus derived therefrom, or a gene delivery vehicle based on said virus its homologue or its chimaera for use as a pharmaceutical.

10   3.   A gene delivery vehicle according to claim 1, whereby said elements comprise adenovirus 35 E3 expression products or the genes encoding them.

4.   A gene delivery vehicle according to claim 1 or 3, whereby said elements comprise adenovirus 35 fiber, penton

15   and/or hexon proteins or a gene encoding either.

5.   A gene delivery according to any one of claims 1,3, or 4 which is a chimaera of adenovirus 35 with at least one other adenovirus.

6.   A gene delivery vehicle according to any one of claims

20   1,3,4 or 5 which has a different tropism than adenovirus 35.

7.   A nucleic acid encoding at least a functional part of a gene delivery vehicle according to any one of claims 1 or 3-6, or a virus, homologue or chimaera thereof according to claim 2.

25   8.   A nucleic acid encoding at least one of the adenovirus serotype 35 elements or a functional equivalent thereof, responsible for avoiding or deminishing neutralising activity against adenoviral elements by the host to which the gene is to be delivered and having a site for introducing a gene of

30   interest therein.

9.   A nucleic acid according to claim 7 or 8, further comprising at least one ITR.

10. A nucleic acid according to claim 7, 8 or 9 further comprising a region of nucleotides designed or useable for homologous recombination.

11. At least one set of two nucleic acids comprising a
5 nucleic acid according to any one of claims 7-10, whereby said set of nucleic acids is capable of a single homologous recombination event with each other, which leads to a nucleic acid encoding a functional gene delivery vehicle.

12. A cell comprising a nucleic acid according to any one of
10 claims 7-10 or a set of nucleic acids according to claim 11.

13. A cell according to claim 12 which complements the necessary elements for adenoviral replication which are absent from the nucleic acid according to any one of claims 7-10 or a set of nucleic acids according to claim 11.

15 14. A cell according to claim 12 or 13, which originates from a PER.C6 cell (ECACC deposit number 96022940).

15. A method for producing a gene delivery vehicle according to claim 1, or 3-6, comprising expressing a nucleic acid according to any one of claims 7-10 in a cell according to
20 claim 12 or 13 and harvesting the resulting gene delivery vehicle.

16. A method for producing a gene delivery vehicle according to claim 1, or 3-6, comprising culturing a cell according to claim 12 or 13 in a suitable culture medium and harvesting
25 the resulting gene delivery vehicle.

17. A gene delivery vehicle obtainable by a method according to claims 15 or 16.

18. A gene delivery vehicle according to any one of claims 1,3-6 or 17, which is derived from a chimaera of an
30 adenovirus and an integrating virus.

19. A gene delivery vehicle according to claim 18, wherein said integrating virus is adeno associated virus.

20. A gene delivery vehicle according to any one of claims 1, 3-6 or 17-19, which has the tropism determining parts of
35 adenovirus 16 or functional equivalents thereof.

21.   A gene delivery vehicle according to anyone of claims 1,
3-6 or 17-20 for use as a pharmaceutical.

22.   A pharmaceutical formulation comprising a gene delivery
vehicle according to any one of claims 1, 3-6 or 17-20 and a
suitable excipient.

23.   A pharmaceutical formulation comprising an adenovirus, a
chimaera thereof, or a functional homologue thereof according
to claim 2 and a suitable excipient.

Figure 1:
% of human sera with neutralising capacity for human adenovirus (n=100)
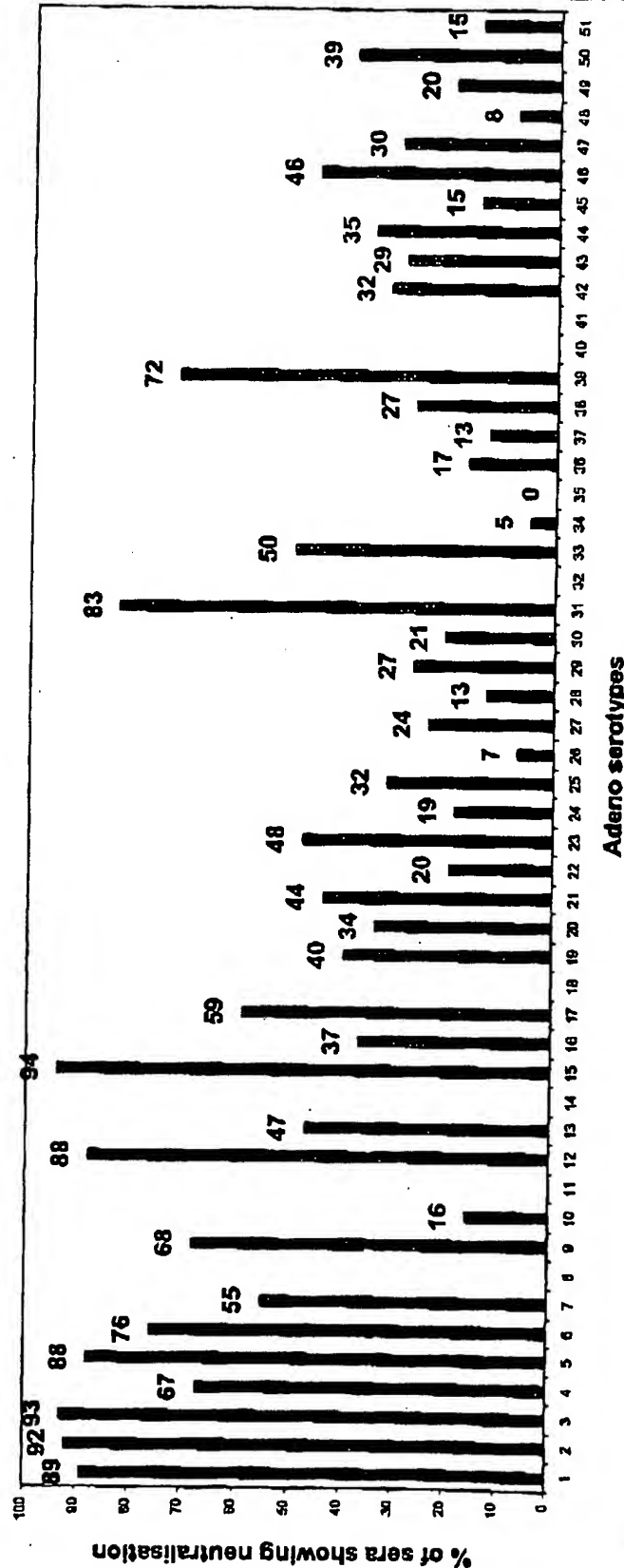
Figure 2
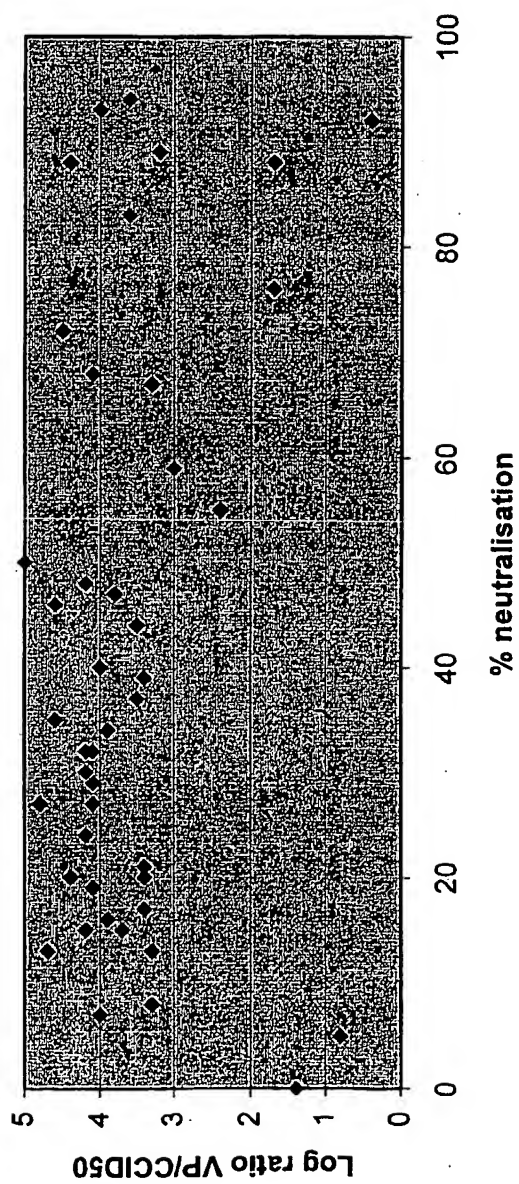
Figure 3: Ad35 plasmid-based system for virus generation
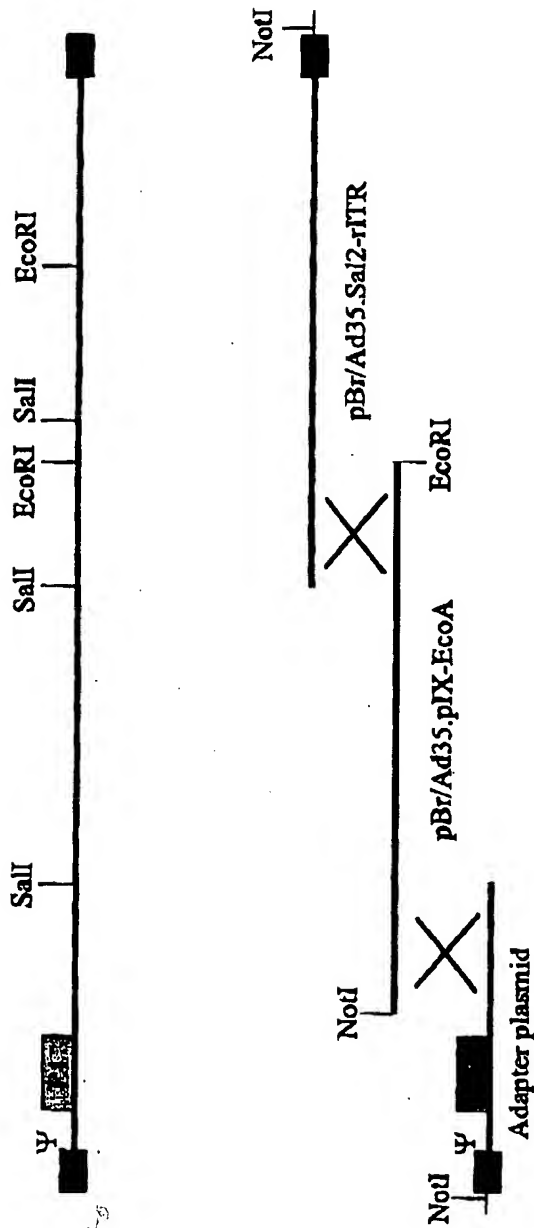
Figure 4

Neutralisation in human sera
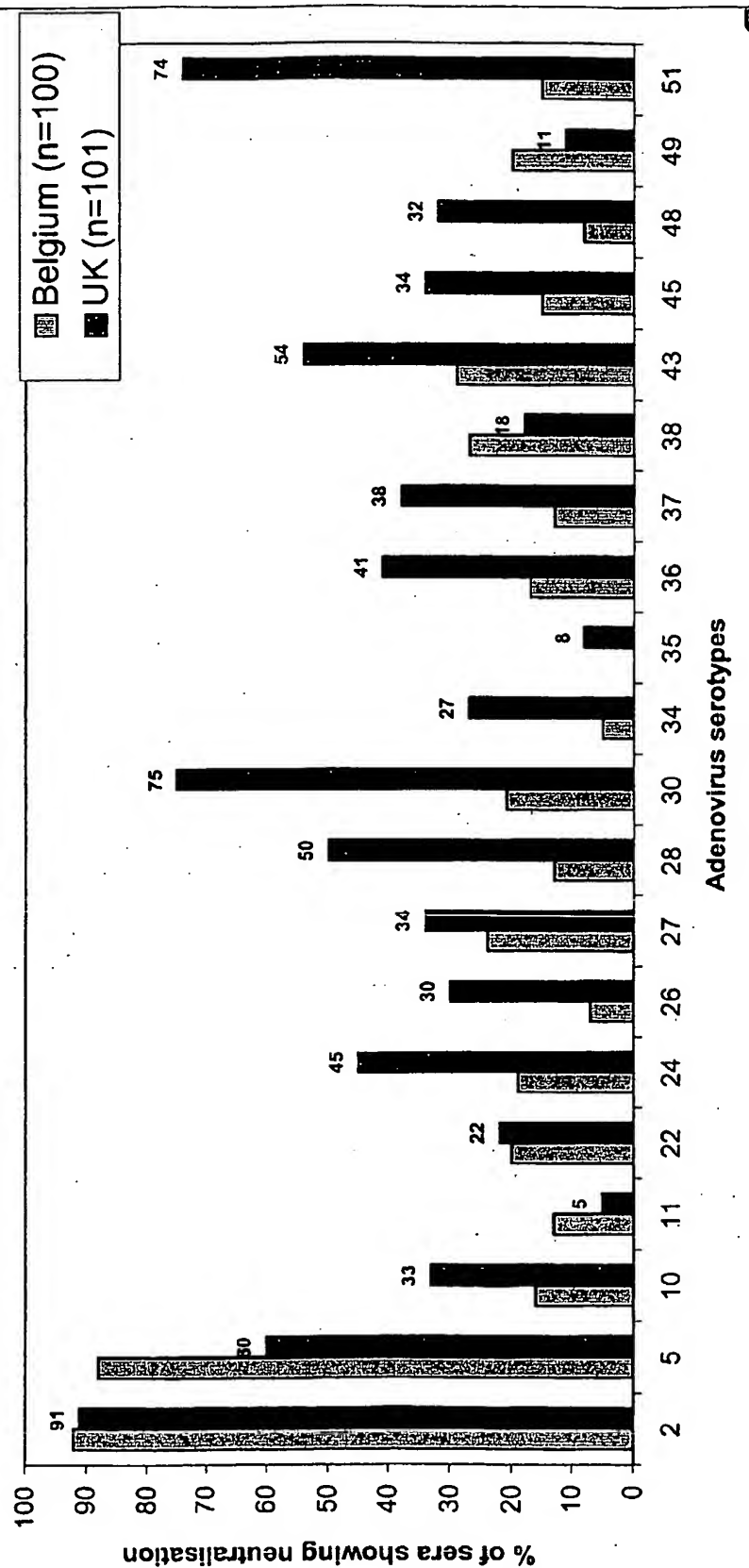
Figure 5

Neutralisation in human sera from different geographic locations

Legend:
- Belgium (n=100)
- UK (n=101)
- US1 (n=100)
- US2 (n=100)
- NL (n=56)

X-axis: Adenovirus serotype (5, 11, 26, 34, 35, 48, 49)
Y-axis: % of sera showing neutralisation (0–100)
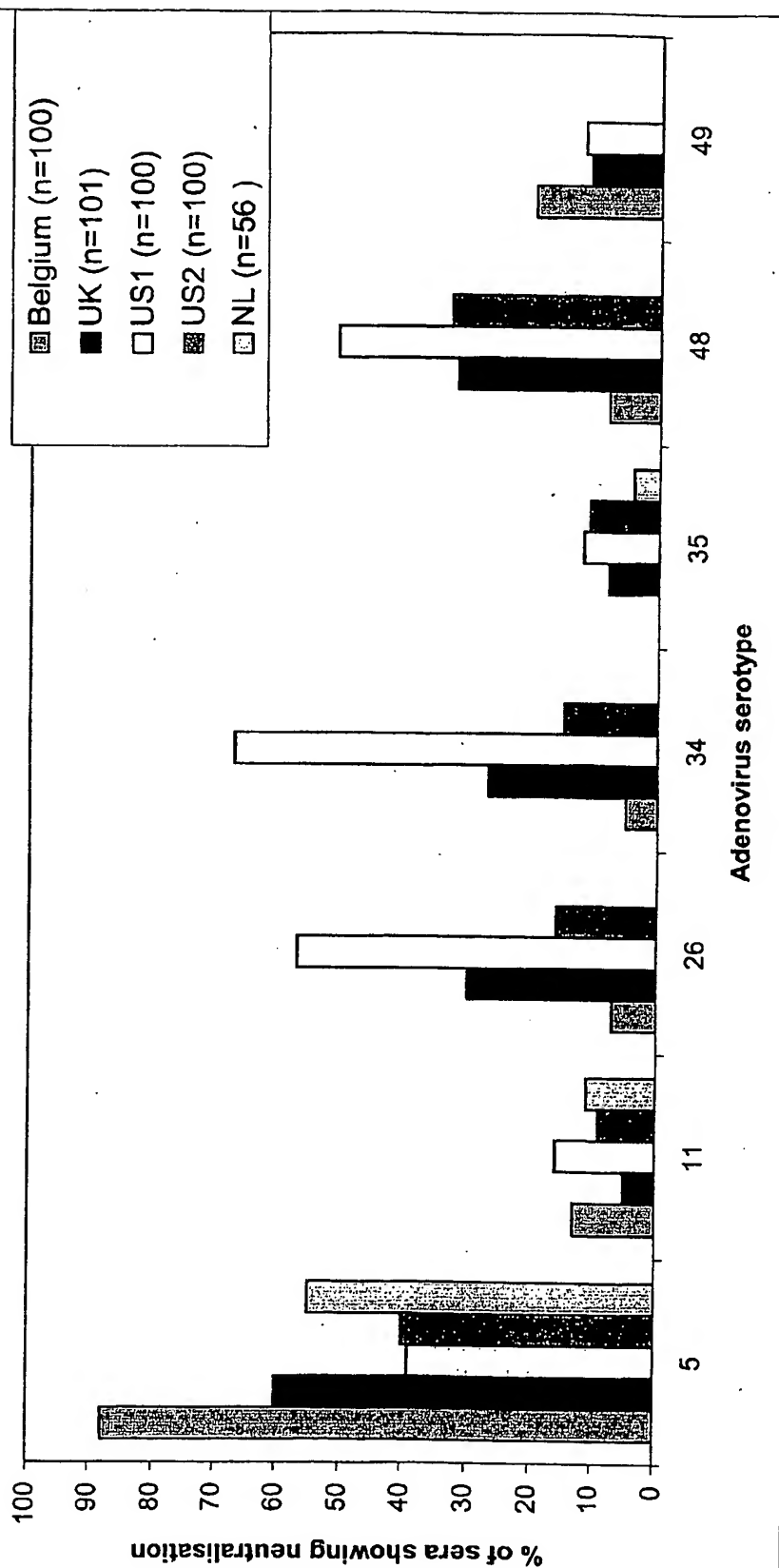
Figure. 6: Total sequence of Ad35.

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   1 CATCATCAAT AATATACCTT ATAGATGGAA TGGTGCCAAT ATGTAAATGA GGTGATTTTA AAAAGTGTGG
  71 GCCGTGTGGT GATTGGCTGT GGGGTTAACG GTTAAAAGGG GCGGCGCGGC CGTGGGAAAA TGACGTTTTA
 141 TGGGGGTGGA GTTTTTTTGC AAGTTGTCGC GGGAAATGTT ACGCATAAAA AGGCTTCTTT TCTCACGGAA
 211 CTACTTAGTT TTCCCACGGT ATTTAACAGG AAATGAGGTA GTTTTGACCG GATGCAAGTG AAAATTGCTG
 281 ATTTTCGCGC GAAAACTGAA TGAGGAAGTG TTTTTCTGAA TAATGTGGTA TTTATGGCAG GGTGGAGTAT
 351 TTGTTCAGGG CCAGGTAGAC TTTGACCCAT TACGTGGAGG TTTCGATTAC CGTGTTTTTT ACCTGAATTT
 421 CCGCGTACCG TGTCAAAGTC TTCTGTTTTT ACGTAGGTGT CAGCTGATCG CTAGGGTATT TATACCTCAG
 491 GGTTTGTGTC AAGAGGCCAC TCTTGAGTGC CAGCGAGAAG AGTTTTCTCC TCTGCGCCGG CAGTTTAATA
 561 ATAAAAAAAT GAGAGATTTG CGATTTCTGC CTCAGGAAAT AATCTCTGCT GAGACTGGAA ATGAAATATT
 631 GGAGCTTGTG GTGCACGCCC TGATGGGAGA CGATCCGGAG CCACCTGTGC AGCTTTTTGA GCCTCCTACG
 701 CTTCAGGAAC TGTATGATTT AGAGGTAGAG GGATCGGAGG ATTCTAATGA GGAAGCTGTG AATGGCTTTT
 771 TTACCGATTC TATGCTTTTA GCTGCTAATG AAGGATTAGA ATTAGATCCG CCTTTGGACA CTTTCAATAC
 841 TCCAGGGGTG ATTGTGGAAA GCGGTACAGG TGTAAGAAAA TTACCTGATT TGAGTTCCGT GGACTGTGAT
 911 TTGCACTGCT ATGAAGACGG GTTTCCTCCG AGTGATGAGG AGGACCATGA AAAGGAGCAG TCCATGCAGA
 981 CTGCAGCGGG TGAGGGAGTG AAGGCTGCCA ATGTTGGTTT TCAGTTGGAT TGCCCGGAGC TTCCTGGACA
1051 TGGCTGTAAG TCTTGTGAAT TTCACAGGAA AAATACTGGA GTAAAGGAAC TGTTATGTTC GCTTTGTTAT
1121 ATGAGAACGC ACTGCCACTT TATTTACAGT AAGTGTGTTT AAGTTAAAAT TTAAAGGAAT ATGCTGTTTT
1191 TCACATGTAT ATTGAGTGTG AGTTTTGTGC TTCTTATTAT AGGTCCTGTG TCTGATGCTG ATGAATCACC
1261 ATCTCCTGAT TCTACTACCT CACCTCCTGA TATTCAAGCA CCTGTTCCTG TGGACGTGCG CAAGCCCATT
1331 CCTGTGAAGC TTAAGCCTGG GAAACGTCCA GCAGTGGAGA AACTTGAGGA CTTGTTACAG GGTGGGGACG
1401 GACCTTTGGA CTTGAGTACA CGGAAACGTC CAAGACAATA AGTGTTCCAT ATCCGTGTTT ACTTAAGGTG
1471 ACGTCAAATAT TTGTGTGAGA GTGCAATGTA ATAAAAATAT GTTAACTGTT CACTGGTTTT TATTGCTTTT
1541 TGGGCGGGGA CTCAGGTATA TAAGTAGAAG CAGACCTGTG TGGTTAGCTC ATAGGAGCTG GCTTTCATCC
1611 ATGGAGGTTT GGGCCATTTT GGAAGACCTT AGGAAGACTA GGCAACTGTT AGAGAGCGCT TCGGACGGAG
1681 TCTCCGGTTT TTGGAGATTC TGGTTCGCTA GTGAATTAGC TAGGGTAGTT TTTAGGATAA AACAGGACTA
1751 TAAACAAGAA TTTGAAAAGT TGTTGGTAGA TTGCCCAGGA CTTTTTGAAG CTCTTAATTT GGGCCATCAG
1821 GTTCACTTTA AAGAAAAAGT TTTATCAGTT TTAGACTTTT CAACCCCAGG TAGAACTGCT GCTGCTGTGG
1891 CTTTTCTTAC TTTTATATTA GATAAATGGA TCCCGCAGAC TCATTTCAGC AGGGGATACG TTTTGGATTT
1961 CATAGCCACA GCATTGTGGA GAACAAGAAA GGTTCGCAAG ATGAGGACAA TCTTAGGTTA CTGGCCAGTG
2031 CAGCCTTTGG GTGTAGCGGG AATCCTGAGG CATCCACCGG TCATGCCAGC GGTTCTGGAG GAGGAACAGC
2101 AAGAGGACAA CCCGAGAGCC GGCCTGGACC CTCCAGTGGA GGAGGCGGAG TAGCTGACTT GTCTCCTGAA
2171 CTGCAACGGG TGCTTACTGG ATCTACGTCC ACTGGACGGG ATAGGGGCGT TAAGAGGGAG AGGGCATCCA
2241 GTGGTACTGA TGCTAGATCT GAGTTGGCTT TAAGTTTAAT GAGTCGCAGA CGTCCTGAAA CCATTTGGTG
2311 GCATGAGGTT CAGAAAGAGG GAAGGGATGA AGTTTCTGTA TTGCAGGAGA AATATTCACT GGAACAGGTG
2381 AAAACATGTT GGTTGGAGCC AGAGGATGAT TGGGCGGTGG CCATTAAAAA TTATGCCAAG ATAGCTTTGA
2451 GGCCTGATAA ACAGTATAAG ATCAGTAGAC GGATTAATAT CCGGAATGCT TGTTACATAT CTGGAAATGG
2521 GGCTGAGGTG GTAATAGATA CTCAAGACAA GACAGTTATT AGATGCTGCA TGATGGATAT GTGGCCTGGA
2591 GTAGTCGGTA TGGAAGCAGT CACTTTTGTA AATGTTAAGT TTAGGGGAGA TGGTTATAAT GGAATAGTGT
2661 TTATGGCCAA TACCAAACTT ATATTGCATG GTTGTAGCTT TTTTGGTTTC AACAATACCT GTGTAGATGC
2731 CTGGGGACAG GTTAGTGTAC GGGGGGTGTAG TTTCTATGCG TGTTGGATTG CCACAGCTGG CAGAACCAAG
2801 AGTCAATTGT CTCTGAAGAA ATGCATATTC CAAAGATGTA ACCTGGGCAT TCTGAATGAA GGCGAAGCAA
2871 GGGTCCGTCA CTGCGCTTCT ACAGATACTG GATGTTTTAT TTTAATTAAG GGAAATGCCA GCGTAAAGCA
2941 TAACATGATT TGTGGTGCTT CCGATGAGAG GCCTTATCAA ATGCTCACTT GTGCTGGTGG GCATTGTAAT
3011 ATGCTGGCTA CTGTGCATAT TGTTTCCCAT CAACGCAAAA AATGGCCTGT TTTTGATCAC AATGTGTTGA
3081 CCAAGTGCAC CATGCATGCA GGTGGGCGTA GAGGAATGTT TATGCCTTAC CAGTGTAACA TGAATCATGT
3151 GAAAGTGTTG TTGGAACCAG ATGCCTTTTC CAGAATGAGC CTAACAGGAA TCTTTGACAT GAACACGCAA
3221 ATCTGGAAGA TCCTGAGGTA TGATGATACG AGATCGAGGG TGCGCGCATG CGAATGCGGA GGCAAGCATG
3291 CCAGGTTCCA GCCGGTGTGT GTAGATGTGA CCGAAGATCT CAGACCGGAT CATTTGGTTA TTGCCCGCAC
3361 TGGAGCAGAG TTCGGATCCA GTGGAGAAGA AACTGACTAA GGTGAGTATT GGGAAAACTT TGGGGTGGGA
3431 TTTTCAGATG GACAGATTGA GTAAAAATTT GTTTTTTCTG TCTTGCAGCT GACATGAGTG GAAATGCTTC
3501 TTTTAAGGGG GGAGTCTTCA GCCCTTATCT GACAGGGCGT CTCCCATCCT GGGCAGGAGT TCGTCAGAAT
3571 GTTATGGGAT CTACTGTGGA TGGAAGACCC GTTCAACCCG CCAATTCTTC AACGCTGACC TATGCTACTT
3641 TAAGTTCTTC ACCTTTGGAC GCAGCTGCAG CCGGCTGCCG CGCCTCTGTC GCCGGTAACA CTGTGCTTGG
3711 AATGGGTTAC TATGGAAGCA TCGTGGCTAA TTCCACTTCC TCTAATAACC CTTCTACACT GACTCAGGAC
3781 AAGTTACTTG TCCTTTTGGC CCAGCTGGAG GCTTTGACCC AACGTCTGGG TGAACTTTCT CAGCAGGTGG
3851 CCGAGTTGCG AGTACAAACT GAGTCTGCTG TCGGCACGGC AAAGTCTAAA TAAAAAAAAT TCCAGAATCA
3921 ATGAATAAAT AAACGAGCTT GTTGTTGATT TAAAATCAAG TGTTTTTATT TCATTTTTCG CGCACGGTAT
3991 GCCCTGGACC ACCGATCTCG ATCATTGAGA ACTCGGTGGA TTTTTTCCAG AATCCTATAG AGGTGGGATT
4061 GAATGTTTAG ATACATGGGC ATTAGGCCGT CTTTGGGGTG GAGATAGCTC CATTGAAGGG ATTCATGCTC
4131 CGGGGTAGTG TTGTAAATCA CCCAGTCATA ACAAGGTCGC AGTGCATGGT GTTGCACAAT ATCTTTTAGA
4201 AGTAGGCTGA TTGCCACAGA TAAGCCCTTG GTGTAGGTGT TTACAAACCG GTTGAGCTGG GAGGGGTGCA
4271 TTCGAGGTGA AATTATGTGC ATTTTGGATT GGATTTTTAA GTTGGCAATA TTGCCGCCAA GATCCCGTCT
```

Figure 6, contd.

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4341 TGGGTTCATG TTATGAAGGA CTACCAAGAC GGTGTATCCG GTACATTTAG GAAATTTATC GTGCAGCTTG
4411 GATGGAAAAG CGTGGAAAAA TTTGGAGACA CCCTTGTGTC CTCCGAGATT TTCCATGCAC TCATCCATGA
4481 TAATAGCAAT GGGGCCGTGG GCAGCGGCGC GGGCAAACAC GTTCCGTGGG TCTGACACAT CATAGTTATG
4551 TTCCTGAGTT AAATCATCAT AAGCCATTTT AATGAATTTG GGGCGGAGCG TACCAGATTG GGGTATGAAT
4621 GTTCCTTCGG GCCCCGGAGC ATAGTTCCCC TCACAGATTT GCATTTCCCA AGCTTTCAGT TCTGAGGGTG
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4761 CAAGTTTCTG AGCAATGAG ATTTGCCACA TCCGGTGGGG CCATAAATAA TTCCGATTAC AGGTTGCAGG
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4901 CATGCATATT TTCCCGCACC AAATCCATTA GGAGGCGCTC TCCTCCTAGT GATAGAAGTT CTTGTAGTGA
4971 GGAAAAGTTT TTCAGCGGTT TTAGACCGTC AGCCATGGGC ATTTTGGAAA GAGTTTGCTG CAAAAGTTCT
5041 AGTCTGTTCC ACAGTTCAGT GATGTGTTCT ATGGCATCTC GATCCAGCAG ACCTCCTCGT TTCGCGGGTT
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5181 CTCAGTGTTC GAGTCAGGGT TGTTTCCGTC ACAGTGAAGG GGTGTGCGCC TGCTTGGGCG CTTGCCAGGG
5251 TGCGCTTCAG ACTCATTCTG CTGGTGGAGA ACTTCTGTCG CTTGGCGCCC TGTATGTCGG CCAAGTAGCA
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5461 AGTATGCATC CGCGCCGCAG GAGGCGCAAA CAGTTTCACA TTCCACCAGC CAGGTTAAAT CCGGTTCATT
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5601 CCTCGTTGAG TGACAAACAG GCTGTCCGTA TCTCCGTAGA CTGATTTTAC AGGCCTCTTC TCCAGTGGAG
5671 TGCCTCGGTC TTCTTCGTAC AGGAACTCTG ACCACTCTGA TACAAAGGCG CGCGTCCAGG CCAGCACAAA
5741 GGAGGCTATG TGGGAGGGGT AGCGATCGTT GTCAACCAGG GGGTCCACCT TTTCCAAAGT ATGCAAACAC
5811 ATGTCACCCT CTTCAACATC CAGGAATGTG ATTGGCTTGT AGGTGTATTT CACGTGACCT GGGGTCCCCG
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5951 CAGCTGTTGG GGTAGGTATT CCCTCTCGAA GGCGGGCATG ACCTCTGCAC TCAGGTTGTC AGTTTCTAAG
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6231 TCGCGTGCCA GGCACTTCCA TTCGGGGAAG ATAGTTGTTA ATTCATCTGG CACGATTCTC ACTTGCCACC
6301 CTCGATTATG CAAGGTAATT AAATCCACAC TGGTGGCCAC CTCGCCTCGA AGGGGTTCAT TGGTCCAACA
6371 GAGCCTACCT CCTTTCCTAG AACAGAAAGG GGGAAGTGGG TCTAGCATAA GTTCATCGGG AGGGTCTGCA
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8681 ACGGCAGCTT GTCTCAGTAT TTCTTGTACG TCACCAGAGT TGTCCTGGTA GGCGATCTCC GCCATGAACT
8751 GCTCGATTTC TTCCTCCTGA AGATCTCCGC GACCCGCTCT TTCGACGGTG GCCGCGAGGT CATTGGAGAT
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Figure 6, contd.

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 8821 ACGGCCCATG AGTTGGGAGA ATGCATTCAT GCCCGCCTCG TTCCAGACGC GGCTGTAAAC CACGGCCCCC
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 8961 TGCATAGGCG CTGAAAAAGG TAGTTGAGTG TGGTGGCAAT GTGTTCGGCG ACGAAGAAAT ACATGATCCA
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 9101 GCAAAATTAA AAAACTGGGA GTTTCGCGCG GACACGGTCA ATTCCTCCTC GAGAAGACGG ATGAGTTCGG
 9171 CTATGGTGGC CCGTACTTCG CGTTCGAAGG CTCCCGGGAT CTCTTCTTCC TCTTCTATCT CTTCTTCCAC
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12951 CTTGCACAGA GCTGCCGCTA TGAACTCTGA TTATTTCACC AATGCCATCT TAAACCCGCA CTGGCTGCCC
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13161 TTCTGCATCG CTGTCCGGGG TCATGGGTGC TACCGCGGCT GAGCCCGAGT CTGCAAGTCC TTTTCCTAGT
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Figure 6, contd.

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13301 AAGAGGAGTA CCTAAACGAT TCCTTGCTCA GACCGGCAAG AGAAAAAAAT TTCCCAAACA ATGGAATAGA
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14841 GTTGGAAGAC AAAATCAACA CAGCCTATCG CAGTTGGTAT CTTTCGTACA ATTATGGCGA TCCCGAAAAA
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Figure 6, contd.

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19111 TGTCATGTAT GCAGAAAATG TAGGTTTGGA AACGCCAGAC ACTCATGTAG TGTACAAACC TGGAACAGAA
19181 GACACAAGTT CCGAAGCTAA TTTGGGACAA CAGTCTATGC CCAACAGACC CAACTACATT GGCTTCAGAG
19251 ATAACTTTAT TGGACTCATG TACTATAACA GTACTGGTAA CATGGGGGTG CTGGCTGGTC AAGCGTCTCA
19321 GTTAAATGCA GTGGTTGACT TGCAGGACAG AAACACAGAA CTTTCTTACC AACTCTTGCT TGACTCTCTG
19391 GGCGACAGAA CCAGATACTT TAGCATGTGG AATCAGGCTG TGGACAGTTA TGATCCTGAT GTACGTGTTA
19461 TTGAAAATCA TGGTGTGGAA GATGAACTTC CCAACTATTG TTTTCCACTG GACGGCATAG GTGTTCCAAC
19531 AACCAGTTAC AAATCAATAG TTCCAAATGG AGAAGATAAT AATAATTGGA AAGAACCTGA AGTAAATGGA
19601 ACAAGTGAGA TCGGACAGGG TAATTTGTTT GCCATGGAAA TTAACCTTCA AGCCAATCTA TGGCGAAGTT
19671 TCCTTTATTC CAATGTGGCT CTGTATCTCC CAGACTCGTA CAAATACACC CCGTCCAATG TCACTCTTCC
19741 AGAAAACAAA AACACCTACG ACTACATGAA CGGGCGGGTG GTGCCGCCAT CTCTAGTAGA CACCTATGTG
19811 AACATTGGTG CCAGGTGGTC TCTGGATGCC ATGGACAATG TCAACCCATT CAACCACCAC CGTAACGCTG
19881 GCTTGCGTTA CCGATCTATG CTTCTGGGTA ACGGACGTTA TGTGCCTTTC CACATACAAG TGCCTCAAAA
19951 ATTCTTCGCT GTTAAAAACC TGCTGCTTCT CCCAGGCTCC TACACTTATG AGTGGAACTT TAGGAAGGAT
20021 GTGAACATGG TTCTACAGAG TTCCCTCGGT AACGACCTGC GGGTAGATGG CGCCAGCATC AGTTTCACGA
20091 GCATCAACCT CTATGCTACT TTTTTCCCCA TGGCTCACAA CACCGCTTCC ACCCTTGAAG CCATGCTGCG
20161 GAATGACACC AATGATCAGT CATTCAACGA CTACCTATCT GCAGCTAACA TGCTCTACCC CATTCCTGCC
20231 AATGCAACCA ATATTCCCAT TTCCATTCCT TCTCGCAACT GGGCGGCTTT CAGAGGCTGG TCATTTACCA
20301 GACTGAAAAC CAAAGAAACT CCCTCTTTGG GGTCTGGATT TGACCCCTAC TTTGTCTATT CTGGTTCTAT
20371 TCCCTACCTG GATGGTACCT TCTACCTGAA CCACACTTTT AAGAAGGTTT CCATCATGTT TGACTCTTCA
20441 GTGAGCTGGC CTGGAAATGA CAGGTTACTA TCTCCTAACG AATTTGAAAT AAAGCGCACT GTGGATGGCG
20511 AAGGCTACAA CGTAGCCCAA TGCAACATGA CCAAAGACTG GTTCTTGGTA CAGATGCTCG CCAACTACAA
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20651 CAGCCCATGA GCAGGCAGGT GGTTGATGAG GTCAATTACA AAGACTTCAA GGCCGTCGCC ATACCCTACC
20721 AACACAACAA CTCTGGCTTT GTGGGTTACA TGGCTCCGAC CATGCGCCAA GGTCAACCCT ATCCCGCTAA
20791 CTATCCCTAT CCACTCATTG GAACAACTGC CGTAAATAGT GTTACGCAGA AAAAGTTCTT GTGTGACAGA
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21211 CCTGGGTTGC GGACCCTATT TTTTGGGAAC CTACGATAAG CGCTTCCCGG GGTTCATGGC CCCCGATAAG
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21351 ACCCACGTTC TAACACCTGC TACCTTTTTG ATCCTTTTGG ATTCTCGGAT GATCGTCTCA AACAGATTTA
21421 CCAGTTTGAA TATGAGGGTC TCCTGCGCCG CAGCGCTCTT GCTACCAAGG ACCGCTGTAT TACGCTGGAA
21491 AAATCTACCC AGACCGTGCA GGGCCCCGT TCTGCCGCCT GCGGACTTTT CTGCTGCATG TTCCTTCACG
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21631 CAACATGCTT CATTCTCCTA AAGTCCAGCC CACCCTGTGT GACAATCAAA AAGCACTCTA CCATTTTCTT
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21771 ATGTTCAATA ATGACTCATG TAAACAACGT GTTCAATAAA CATCACTTTA TTTTTTTACA TGTATCAAGG
21841 CTCTGGATTA CTTATTTATT TACAAGTCGA ATGGGTTCTG ACGAGAATCA GAATGACCCG CAGGCAGTGA
21911 TACGTTGCGG AACTGATACT TGGGTTGCCA CTTGAATTCG GGAATCACCA ACTTGGGAAC CGGTATATCG
21981 GGCAGGATGT CACTCCACAG CTTTCTGGTC AGCTGCAAAG CTCCAAGCAG GTCAGGAGCC GAAATCTTGA
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22121 CAGCGACGGA TGTCTCACGC TTGCCAGCAC GGTGGGATCT GCAATCATGC CCACATCCAG ATCTTCAGCA
22191 TTGGCAATGC TGAACGGGGT CATCTTGCAG GTCTGCCTAC CCATGGCGGG CACCCAATTA GGCTTGTGGT
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Figure 6, contd.

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22261 TGCAATCGCA GTGCAGGGGG ATCAGTATCA TCTTGGCCTG ATCCTGTCTG ATTCCTGGAT ACACGGCTCT
22331 CATGAAAGCA TCATATTGCT TGAAAGCCTG CTGGGCTTTA CTACCCTCGG TATAAAACAT CCCGCAGGAC
22401 CTGCTCGAAA ACTGGTTAGC TGCACAGCCG GCATCATTCA CACAGCAGCG GGCGTCATTG TTGGCTATTT
22471 GCACCACACT TCTGCCCCAG CGGTTTTGGG TGATTTTGGT TCGCTCGGGA TTCTCCTTTA AGGCTCGTTG
22541 TCCGTTCTCG CTGGCCACAT CCATCTCGAT AATCTGCTCC TTCTGAATCA TAATATTGCC ATGCAGGCAC
22611 TTCAGCTTGC CCTCATAATC ATTGCAGCCA TGAGGCCACA ACGCACAGCC TGTACATTCC CAATTATGGT
22681 GGGCGATCTG AGAAAAAGAA TGTATCATTC CCTGCAGAAA TCTTCCCATC ATCGTGCTCA GTGTCTTGTG
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22891 GACACATCAC TTCCATGCCT TTCTCCCAAG CAGACACCAG GGGCAAGCTA ATCGGATTCT TAACAGTGCA
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23101 CTTGACTGAT GTCTTGCATG GGGATATGTT TGGTCTTCCT TGGCTTCTTT TTGGGGGGTA TCGGAGGAGG
23171 AGGACTGTCG CTCCGTTCCG GAGACAGGGA GGATTGTGAC GTTTCGCTCA CCATTACCAA CTGACTGTCG
23241 GTAGAAGAAC CTGACCCCAC ACGGCGCACG GTGTTTTTCT TCGGGGGCAG AGGTGGAGGC GATTGCGAAG
23311 GGCTGCGGTC CGACCTGGAA GGCGGATGAC TGGCAGAACC CCTTCCGCGT TCGGGGGTGT GCTCCCTGTG
23381 GCGGTCGCTT AACTGATTTC CTTCGCGGCT GGCCATTGTG TTCTCCTAGG CAGAGAAACA ACAGACATGG
23451 AAACTCAGCC ATTGCTGTCA ACATCGCCAC GAGTGCCATC ACATCTCGTC CTCAGCGACG AGGAAAAGGA
23521 GCAGAGCTTA AGCATTCCAC CGCCCAGTCC TGCCACCACC TCTACCCTAG AAGATAAGGA GGTCGACGCA
23591 TCTCATGACA TGCAGAATAA AAAAGCGAAA GAGTCTGAGA CAGACATCGA GCAAGACCCG GGCTATGTGA
23661 CACCGGTGGA ACACGAGGAA GAGTTGAAAC GCTTTCTAGA GAGAGAGGAT GAAAACTGCC CAAAACAGCG
23731 AGCAGATAAC TATCACCAAG ATGCTGGAAA TAGGGATCAG AACACCGACT ACCTCATAGG GCTTGACGGG
23801 GAAGACGCGC TCCTTAAACA TCTAGCAAGA CAGTCGCTCA TAGTCAAGGA TGCATTATTG GACAGAACTG
23871 AAGTGCCCAT CAGTGTGGAA GAGCTCAGCT GCGCCTACGA GCTTAACCTT TTTTCACCTC GTACTCCCCC
23941 CAAACGTCAG CCAAACGGCA CCTGCGAGCC AAATCCTCGC TTAAACTTTT ATCCAGCTTT TGCTGTGCCA
24011 GAAGTACTGG CTACCTATCA CATCTTTTTT AAAAATCAAA AAATTCCAGT CTCCTGCCGC GCTAATCGCA
24081 CCCGCGCCGA TGCCCTACTC AATCTGGGAC CTGGTTCACG CTTACCTGAT ATAGCTTCCT TGGAAGAGGT
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24291 GAAGCGTCGA GGTCACACAC TTCGCATATC CCGCTGTCAA CCTGCCCCCT AAAGTCATGA CGGCGGTCAT
24361 GGACCAGTTA CTCATTAAGC GCGCAAGTCC CCTTTCAGAA GACATGCCATG ACCCAGATGC CTGTGATGAG
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24501 AGCGTCGCAA GCTTATGATG GCCGTGGTGC TGGTTACCGT AGAACTAGAG TGTCTCCGAC GTTTCTTTAC
24571 CGATTCAGAA ACCTTGCGCA AACTCGAAGA GAATCTGCAC TACACTTTTA GACACGGCTT TGTGCGGCAG
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25131 GCTACCTGTT CCGTCTGGCC AACTATCTCT CCTACCACTC GGATGTGATC GAGGATGTGA GCGGAGACGG
25201 CTTGCTGGAG TGCCGATGCC GCTGCAATCT GTGCACGCCC CACCGGTCCC TAGCTTGCAA CCCCCAGTTG
25271 ATGAGCGAAA CCCAGATAAT AGGCACCTTT GAATTGCAAG GCCCCAGCAG CCAAGGCGAT GGGTCTTCTC
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25621 CCCAACGACG AGAAAACAAG AAGTTGAAGG TGCAGCCGCC GCCCCAGAA GATATGGAGG AAGATTGGGA
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25971 GGGCATAAGA ATGCCATCAT CTCCTGCTTG CATGAGTGCG GGGGCAACAT ATCCTTCACG CGGCGCTACT
26041 TGCTATTCCA CCATGGGGTG AACTTTCCGC GCAATGTTTT GCATTACTAC CGTCACCTCC ACAGCCCCTA
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26181 AGCGGCAGTT AGAAAATACA CAACAAGTGC AGCAACAGGA GGATTAAAGA TTACAGCCAA CGAGCCAGCG
26251 CAAACCCGAG AGTTAAGAAA TCGGATCTTT CCAACCCTGT ATGCCATCTT CCAGCAGAGT CGGGGTCAAG
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26391 AGATCAACTT CAGCGCACTC TCGAGGACGC CGAGGCTCTC TTCAACAAGT ACTGCGCGCT GACTCTTAAA
26461 GAGTAGGCAG CGACCGCGCT TATTCAAAAA AGGCGGGAAT TACATCATCC TCGACATGAG TAAAGAAATT
26531 CCCACGCCTT ACATGTGGAG TTATCAACCC CAAATGGGAT TGGCAGCAGG CGCCTCCCAG GACTACTCCA
26601 CCCGCATGAA TTGGCTCAGC GCCGGGCCTT CTATGATTTC TCGAGTTAAT GATATACGCG CCTACCGAAA
26671 CCAAATACTT TTGGAACAGT CAGCTCTTAC CACCACGCCC CGCCAACACC TTAATCCCAG AAATTGGCCC
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Figure 6, contd.

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26951 CTACGACCAG ACGGAATCTT TCAGATTGCC GGCTGCGGGA GATCTTCCTT CACCCCTCGT CAGGCTGTTC
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27301 CTCCCCAAGG ATCACCCTCA AGGTCCGGCC CACGGAGTGC GGATTACTAT CGAAGGCAAA ATAGACTCTC
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27931 GACCCAGAAA ACTGCACACT TACTTTTGCA CCCGACACAA GCCGCATCTG TGGAGTTCTT ATTAAGTGCG
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28421 GGACAAACAG TCGTCTCTAT CCCACTAGGA CATAATTACA CTCTCATAGG ACCCCCAATC ACTTCAGAGG
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28561 AACTTGCAAC ATACAAAATC TTACATTGAT TAATGTTAGC AAAGTTTACA GCGGTTACTA TTATGGTTAT
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29331 GAATAATTCT ACAACTTCAC ATACAACAAT TTCCACTTCA ACAATCAGCA TCATCGCTGC AGTGACAATT
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29471 ATCCATTACT TAGATTTGAT ATTTAATTTG TTCTTTTTTT TTATTTACAG TATGGTGAAC ACCAATCATG
29541 GTACCTAGAA ATTTCTTCTT CACCATACTC ATCTGTGCTT TTAATGTTTG CGCTACTTTC ACAGCAGTAG
29611 CCACAGCAAC CCCAGACTGT ATAGGAGCAT TTGCTTCCTA TGCACTTTTT GCTTTTGTTA CTTGCATCTG
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29751 TACCTGCGCC ACCATCCCGA ATACCGCAAC CAAAATATCG CGGCACTTCT TAGACTCATC TAAAACCATG
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30311 CAGCAGCAGG AACGCGTGGC CAAAGAGCTC AGAGATGTCA TCCAAATTCA CCAATGCAAA AAAGGCATAT
30381 TCTGTTTGGT AAAACAAGCC AAGATATCCT ACGAGATCAC CGCTACTGAC CATCGCCTCT CTTACGAACT
30451 TGGCCCCCAA CGACAAAAAT TTACCTGCAT GGTGGGAATC AACCCCATAG TTATCACCCA ACAAAGTGGA
30521 GATACTAAGG GTTGCATTCA CTGCTCCTGC GATTCCATCG AGTGCACCTA CACCCTGCTG AAGACCCTAT
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30661 GCAATAAGGT CTCTGTTGAA ATTTTCTCCC AGCAGCACCT CACTTCCCTC TTCCCAACTC TGGTATTCTA
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30801 CACAATCTTC ATGTCTTTCT TCCCAGATGA CCAAGAGAGT CCGGCTCAGT GACTCCTTCA ACCCTGTCTA
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31081 AGCACCCATT ACTAAAAATA ATCACTCTGT AGAACTATCC ATTGGAAATG GATTAGAAAC TCAAAACAAT
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Figure 6, contd.

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31361 GACACTGTGA ACCAAATGTT CACACAAAAG ACAGCAAACA TCCAATTAAG ATTATATTTT GACTCTTCTG
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32201 CACAGTTTCC TGAAGCATGA TTTTAATAGC CCTTAACATC AACTTTCTGG TGCGATGCGC GCAGCAACGC
32271 ATTCTGATTT CACTCAAATC TTTGCAGTAG GTACAACACA TTATTACAAT ATTGTTTAAT AAACCATAAT
32341 TAAAAGCGCT CCAGCCAAAA CTCATATCTG ATATAATCGC CCCTGCATGA CCATCATACC AAAGTTTAAT
32411 ATAAATTAAA TGACGTTCCC TCAAAAACAC ACTACCCACA TACATGATCT CTTTTGGCAT GTGCATATTA
32481 ACAATCTGTC TGTACCATGG ACAACGTTGG TTAATCATGC AACCCAATAT AACCTTCCGG AACCACACTG
32551 CCAACACCGC TCCCCCAGCC ATGCATTGAA GTGAACCCTG CTGATTACAA TGACAATGAA GAACCCAATT
32621 CTCTCGACCG TGAATCACTT GAGAATGAAA AATATCTATA GTGGCACAAC ATAGACATAA ATGCATGCAT
32691 CTTCTCATAA TTTTTAACTC CTCAGGATTT AGAAACATAT CCCAGGGAAT AGGAAGCTCT TGCAGAACAG
32761 TAAAGCTGGC AGAACAAGGA AGACCACGAA CACAACTTAC ACTATGCATA GTCATAGTAT CACAATCTGG
32831 CAACAGCGGG TGGTCTTCAG TCATAGAAGC TCGGGTTTCA TTTTCCTCAC AACGTGGTAA CTGGGCTCTG
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33741 TAGCCAGAAG CCCCCCGGGA ACAAGAGCAG GGGACGCTAC AGTGCAGTAC AAGCGCAGAC CTCCCCAATT
33811 GGCTCCAGCA AAAACAAGAT TGGAATAAGC ATATTGGGAA CCACCAGTAA TATCATCGAA GTTGCTGGAA
33881 ATATAATCAG GCAGAGTTTC TTGTAGAAAT TGAATAAAAG AAAAATTTGC CAAAAAAACA TTCAAAACCT
33951 CTGGGATGCA AATGCAATAG GTTACCGCGC TGCGCTCCAA CATTGTTAGT TTTGAATTAG TCTGCAAAAA
34021 TAAAAAAAAA ACAAGCGTCA TATCATAGTA GCCTGACGAA CAGGTGGATA AATCAGTCTT TCCATCACAA
34091 GACAAGCCAC AGGGTCTCCA GCTCGACCCT CGTAAAACCT GTCATCGTGA TTAAACAACA GCACCGAAAG
34161 TTCCTCGCGG TGACCAGCAT GAATAAGTCT TGATGAAGCA TACAATCCAG ACATGTTAGC ATCAGTTAAG
34231 GAGAAAAAAC AGCCAACATA GCCTTTGGGT ATAATTATGC TTAATCGTAA GTATAGCAAA GCCACCCCTC
34301 GCGGATACAA AGTAAAAGGC ACAGGAGAAT AAAAAAATATA ATTATTTCTC TGCTGCTGTT TAGGCAACGT
34371 CGCCCCCGGT CCCTCTAAAT ACACATACAA AGCCTCATCA GCCATGGCTT ACCAGAGAAA GTACAGCGGG
34441 CACACAAACC ACAAGCTCTA AAGTCACTCT CCAACCTSTC CACAATATAT ATACACAAGC CCTAAACTGA
34511 CGTAATGGGA CTAAAGTGTA AAAAATCCCG CCAAACCCAA CACACACCCC GAAACTGCGT CACCAGGGAA
34581 AAGTACAGTT TCACTTCCGC AATCCCAACA AGCGTCACTT CCTCTTTCTC ACGGTACGTC ACATCCCATT
34651 AACTTACAAC GTCATTTTCC CACGGCCGCG CCGCCCCTTT TAACCGTTAA CCCCACAGCC AATCACCACA
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34791 GATG
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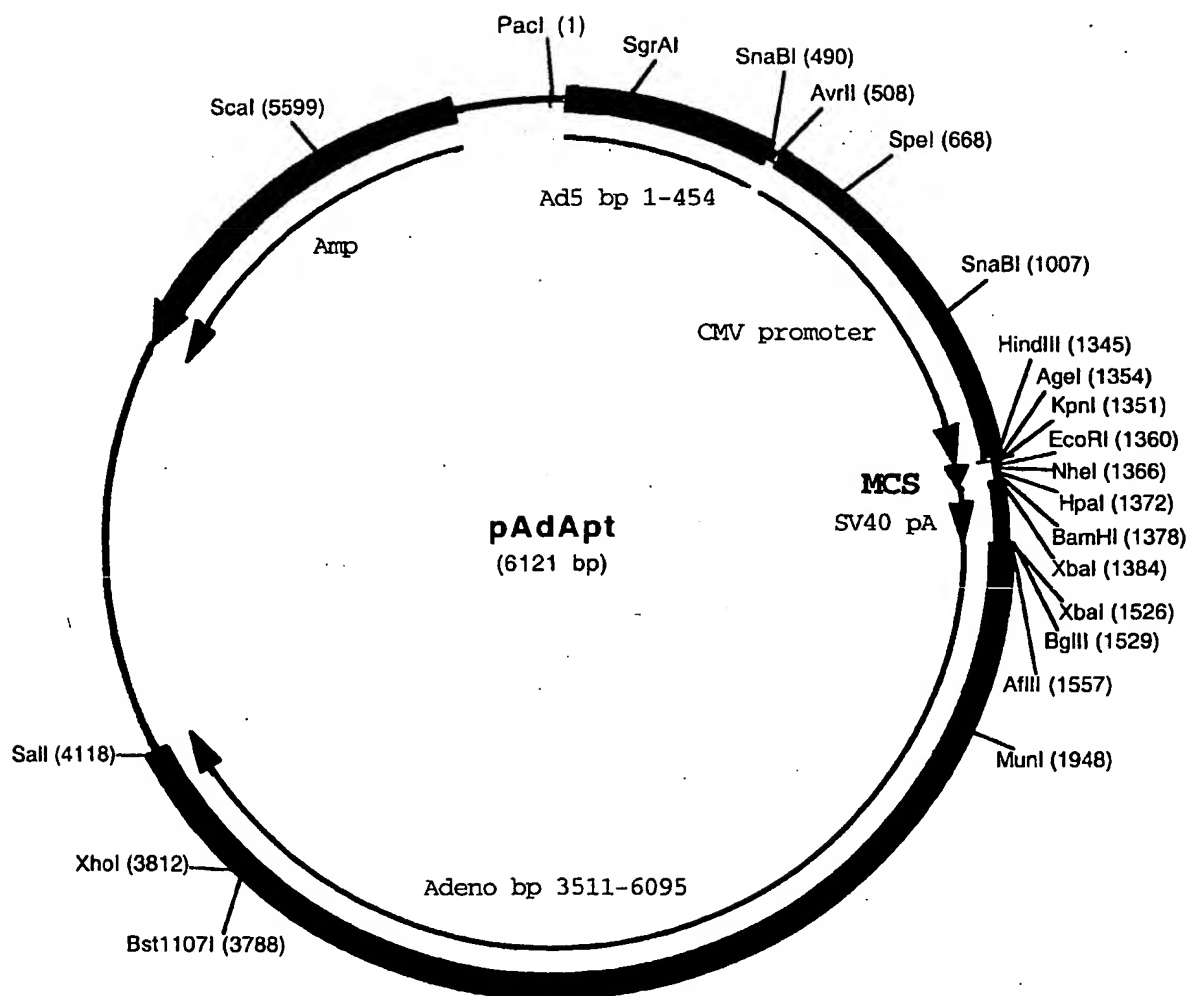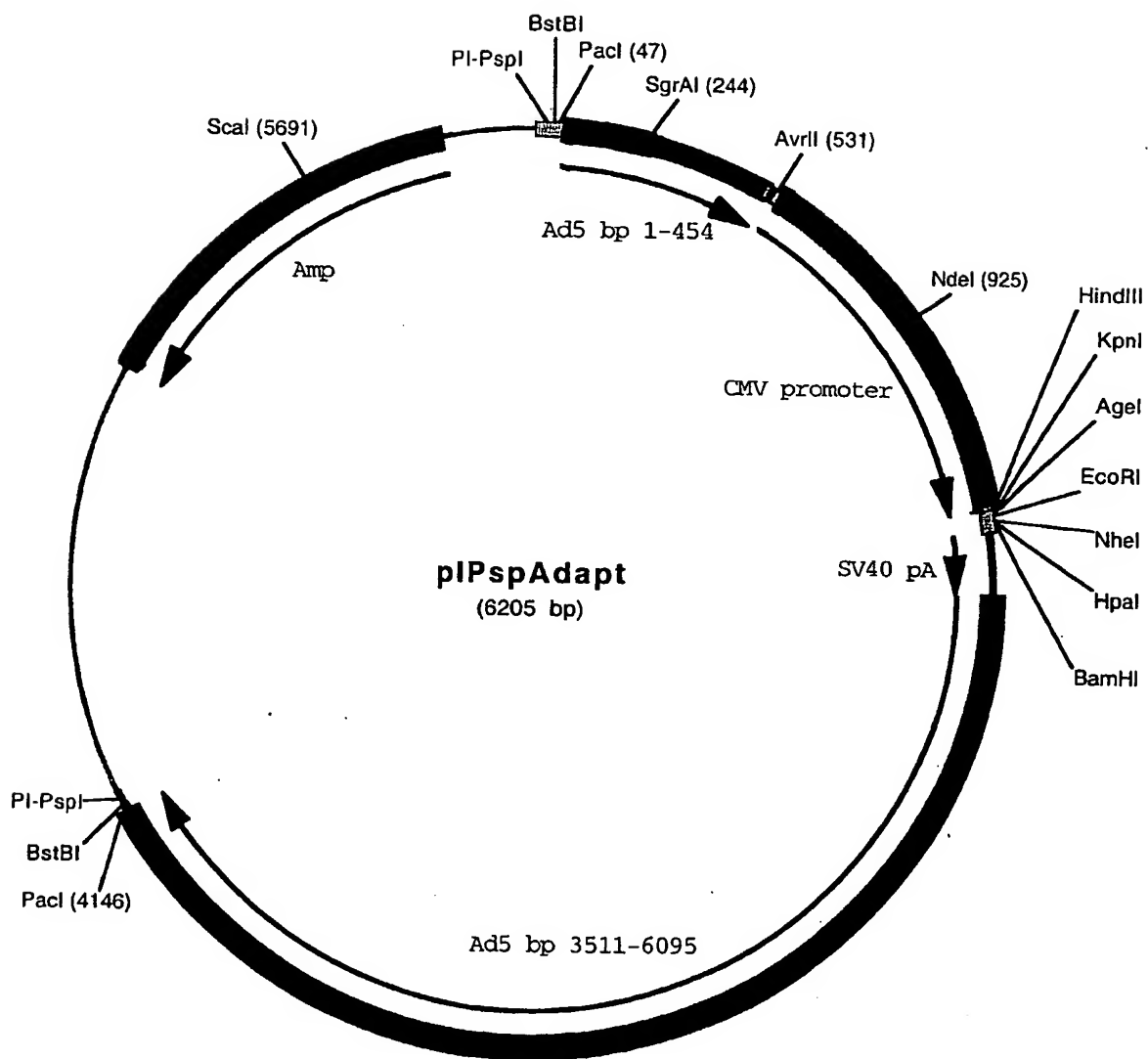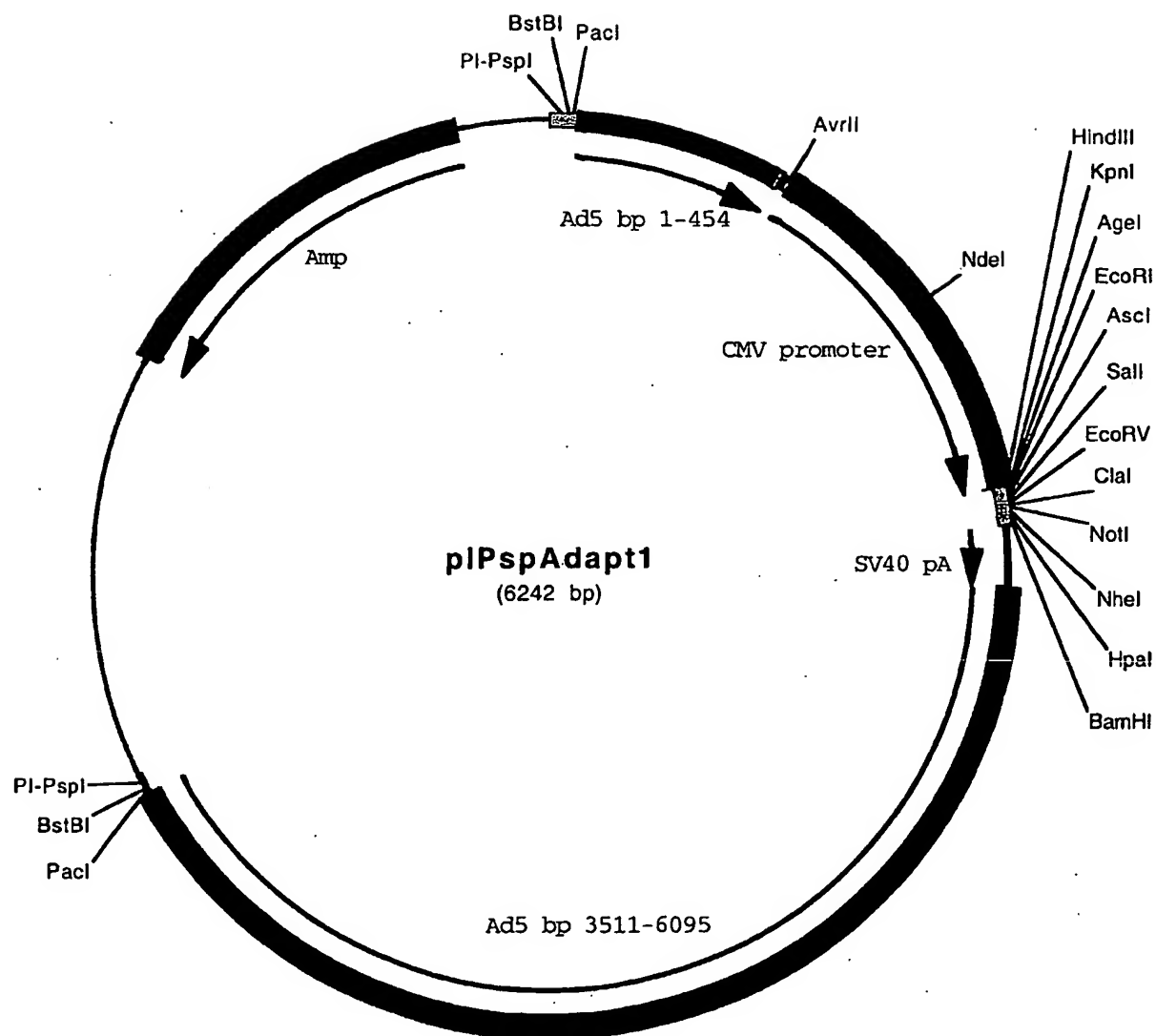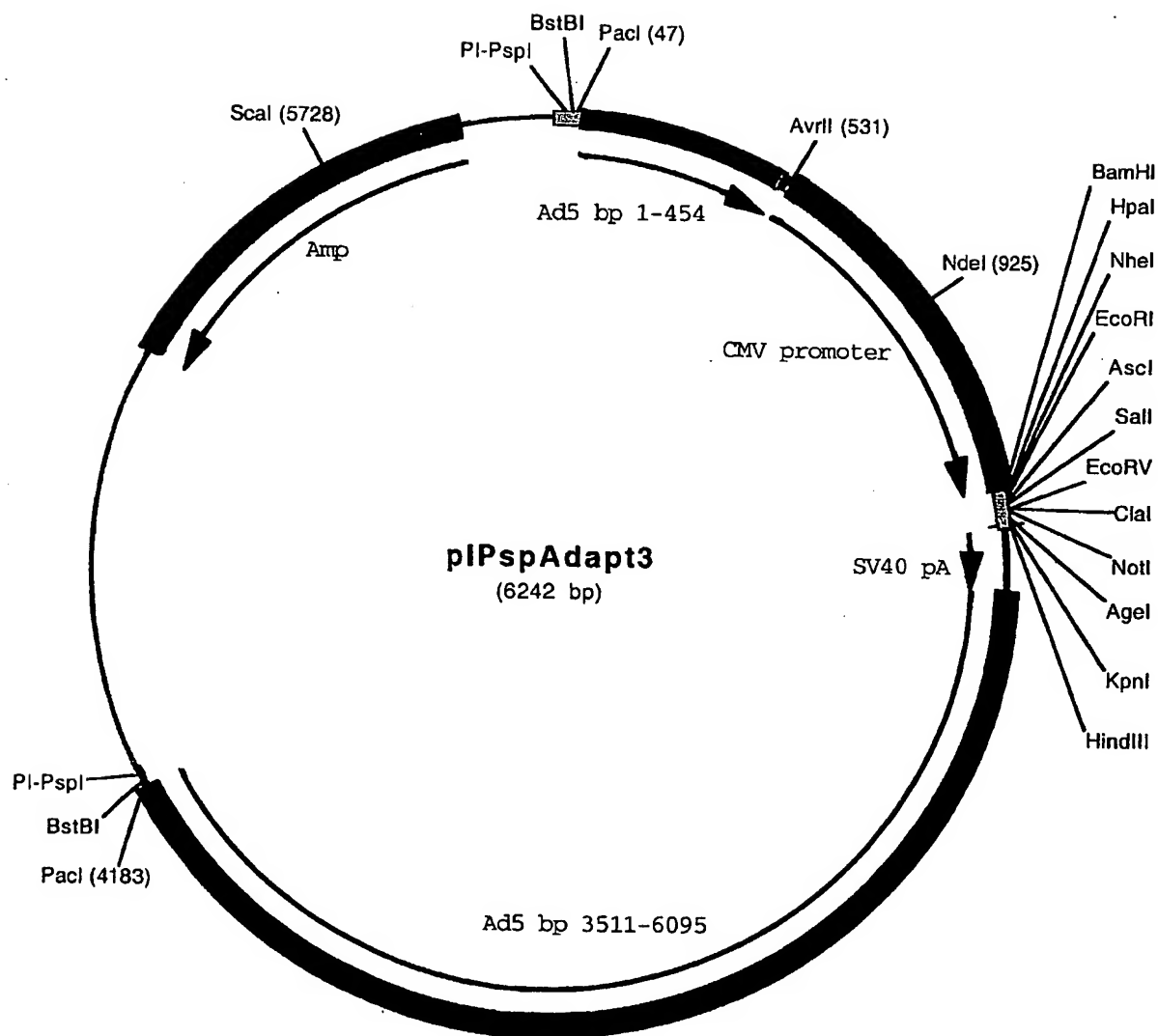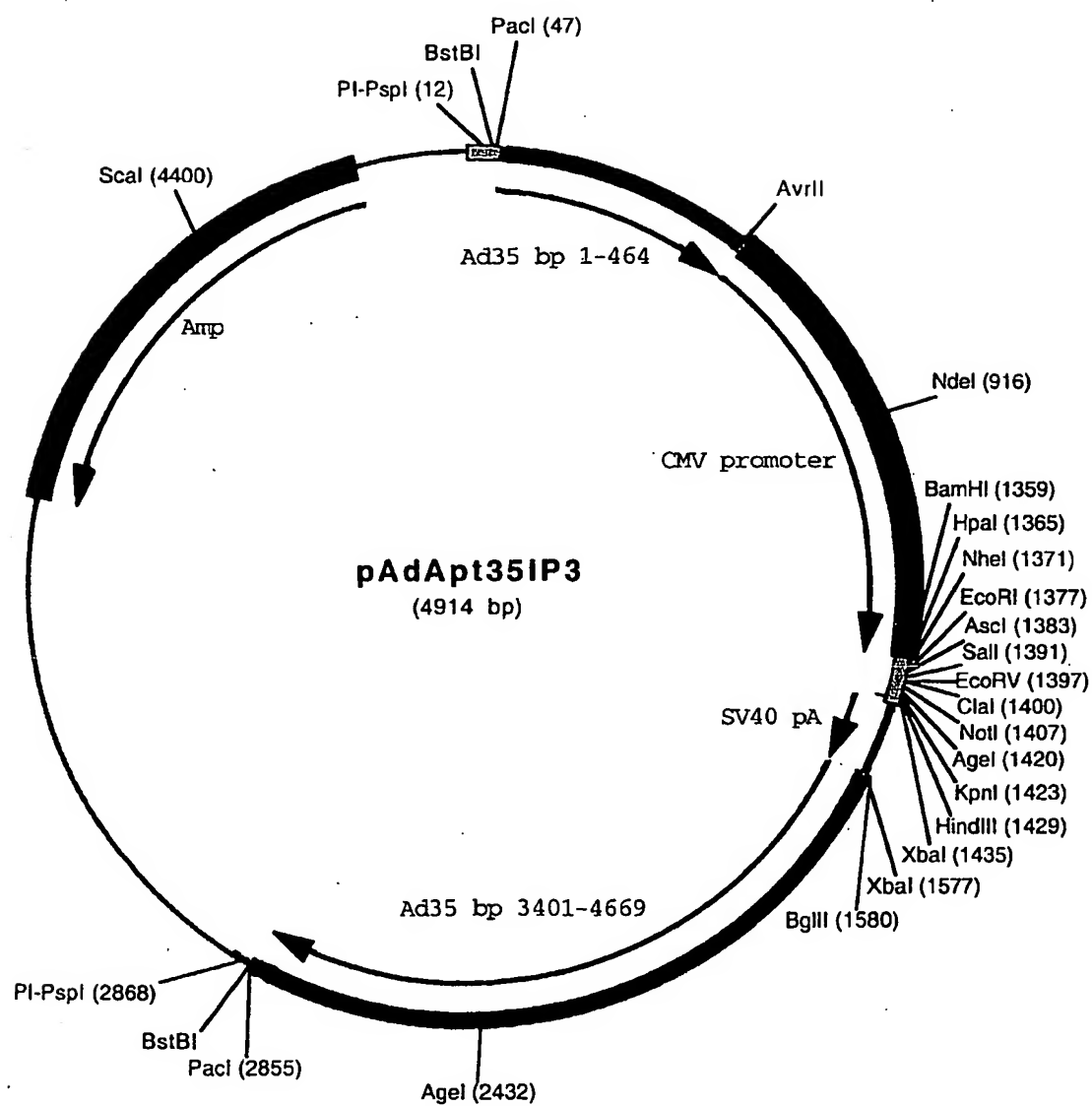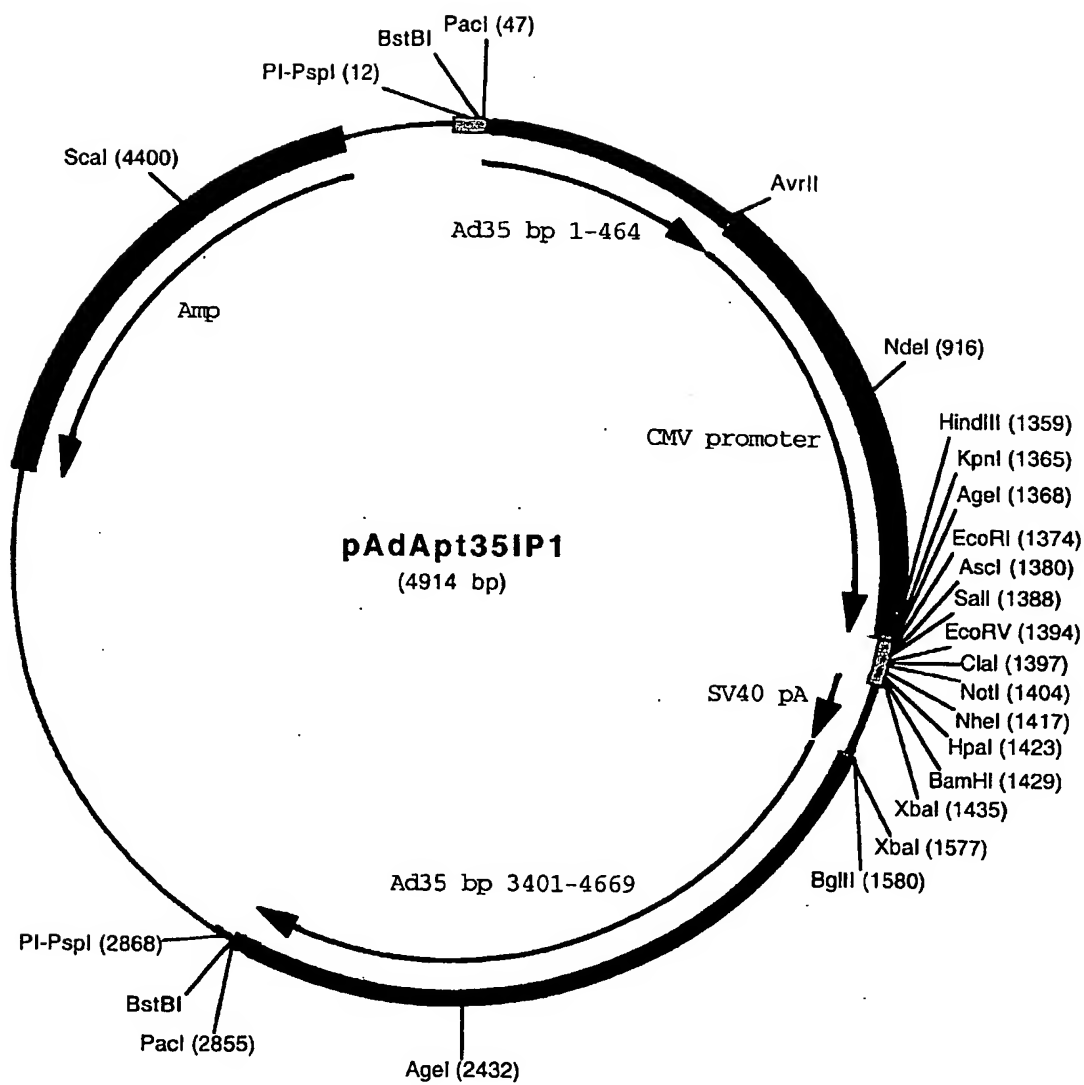
Figure 7

Figure 8

Figure 9

Figure 10

Figure 11
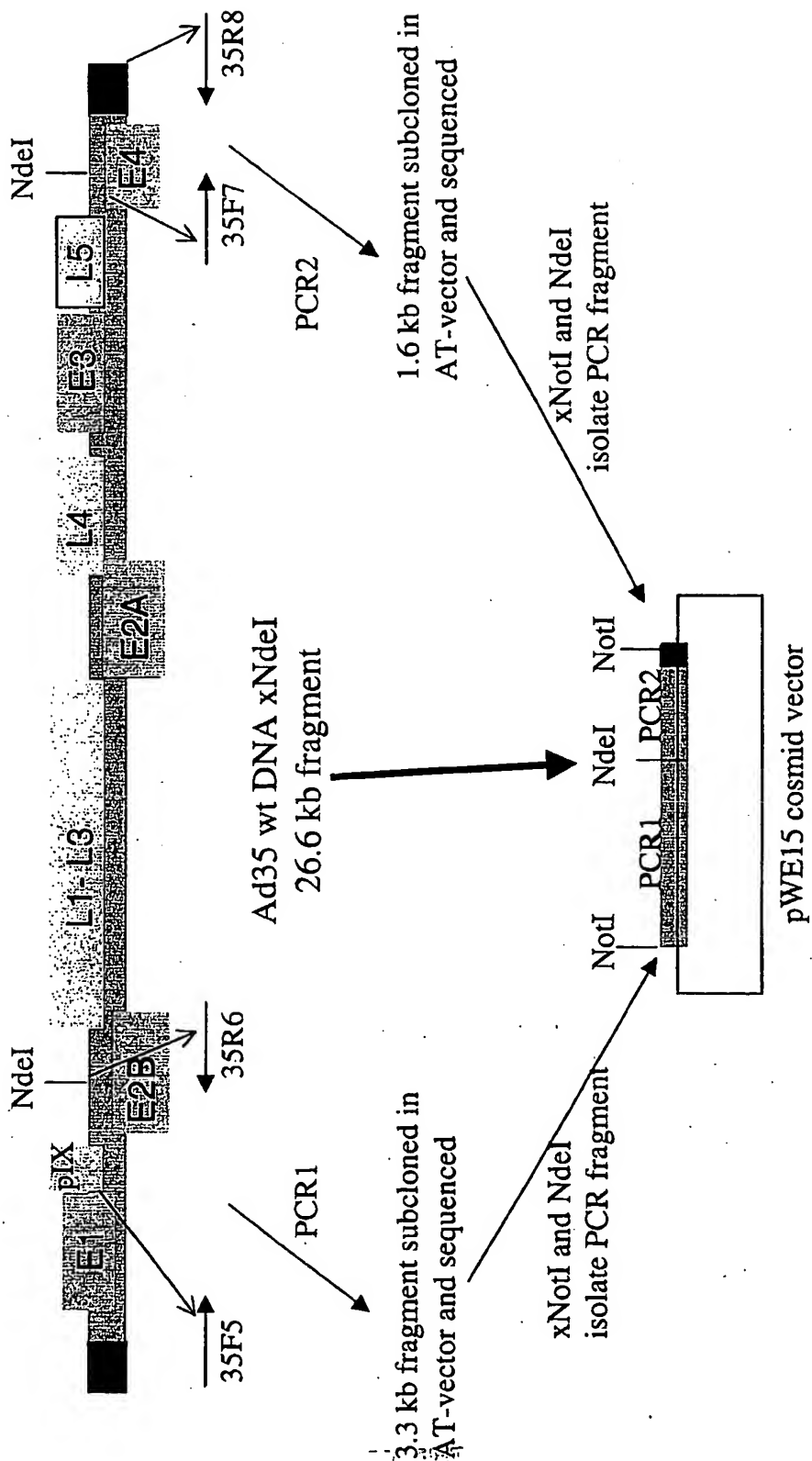
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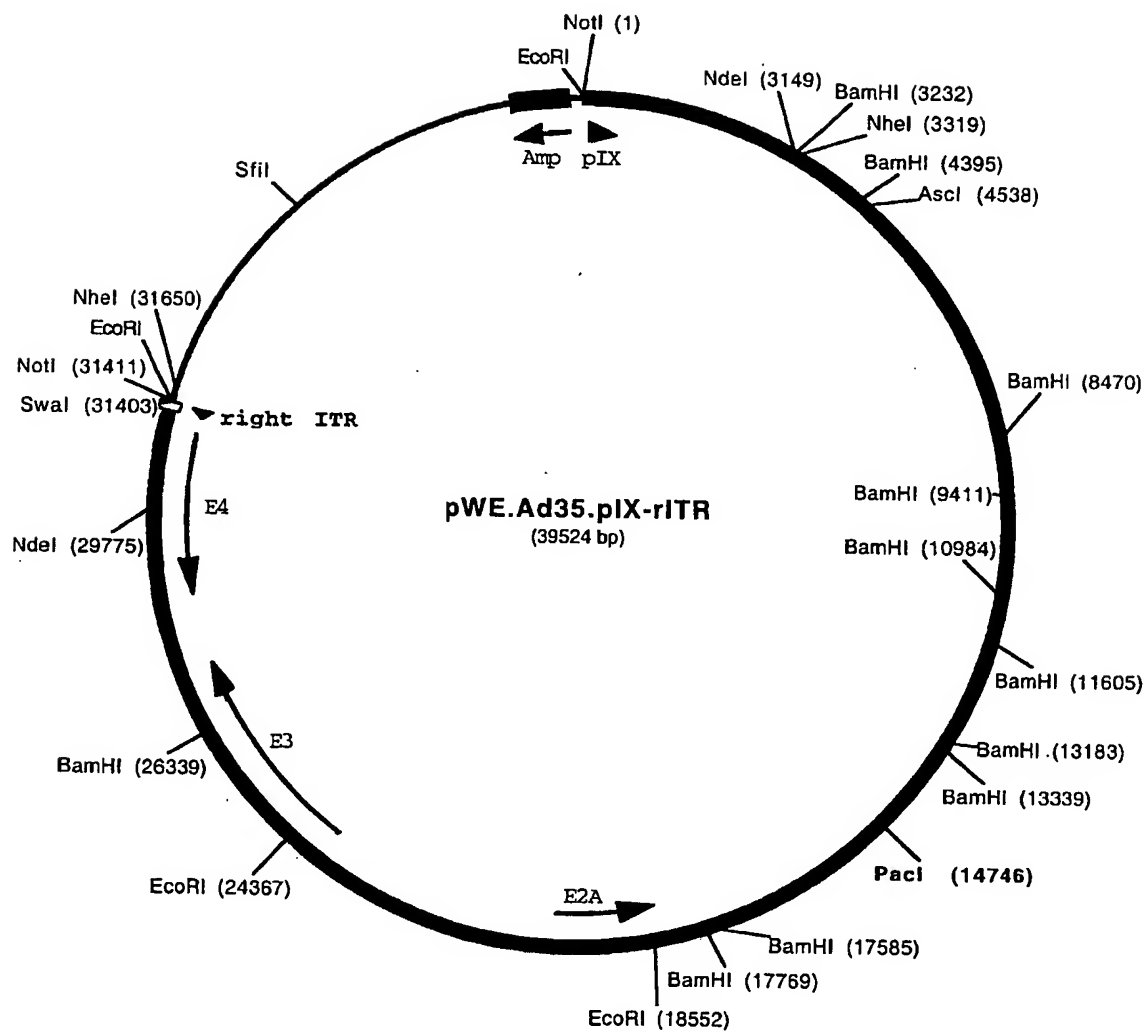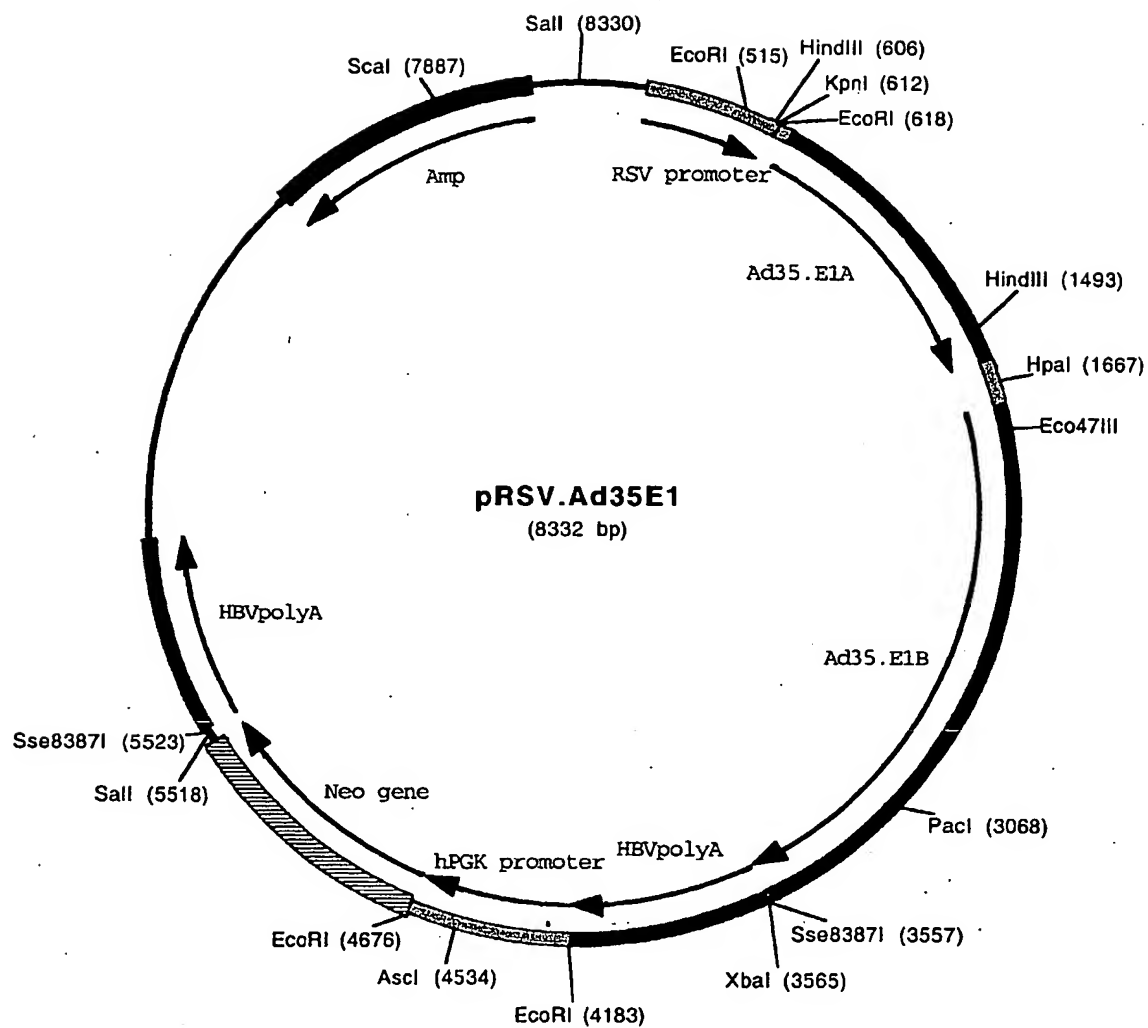Construction of cosmid vector pWE.Ad35.pIX-rITR

Figure 13

Figure 14

Figure 15

Figure 16
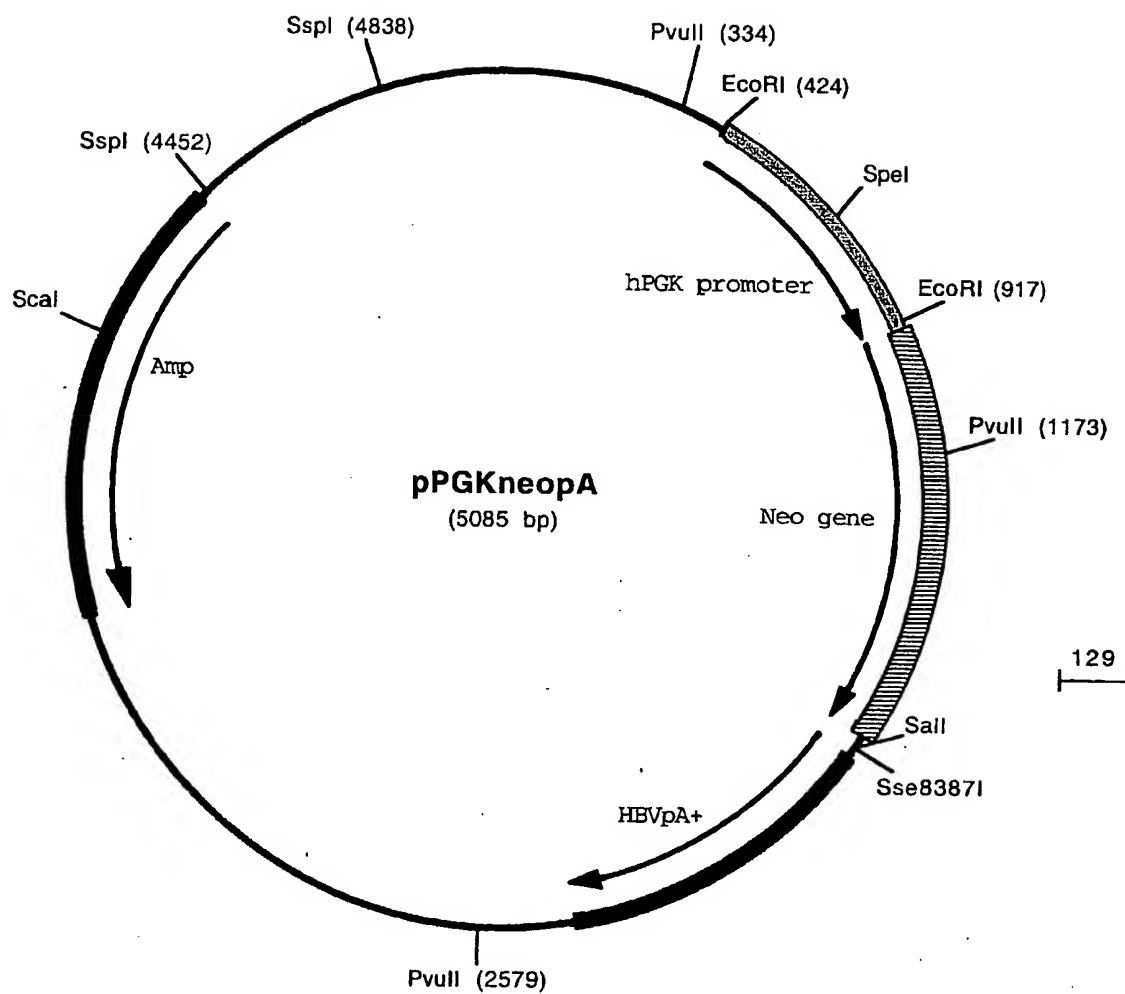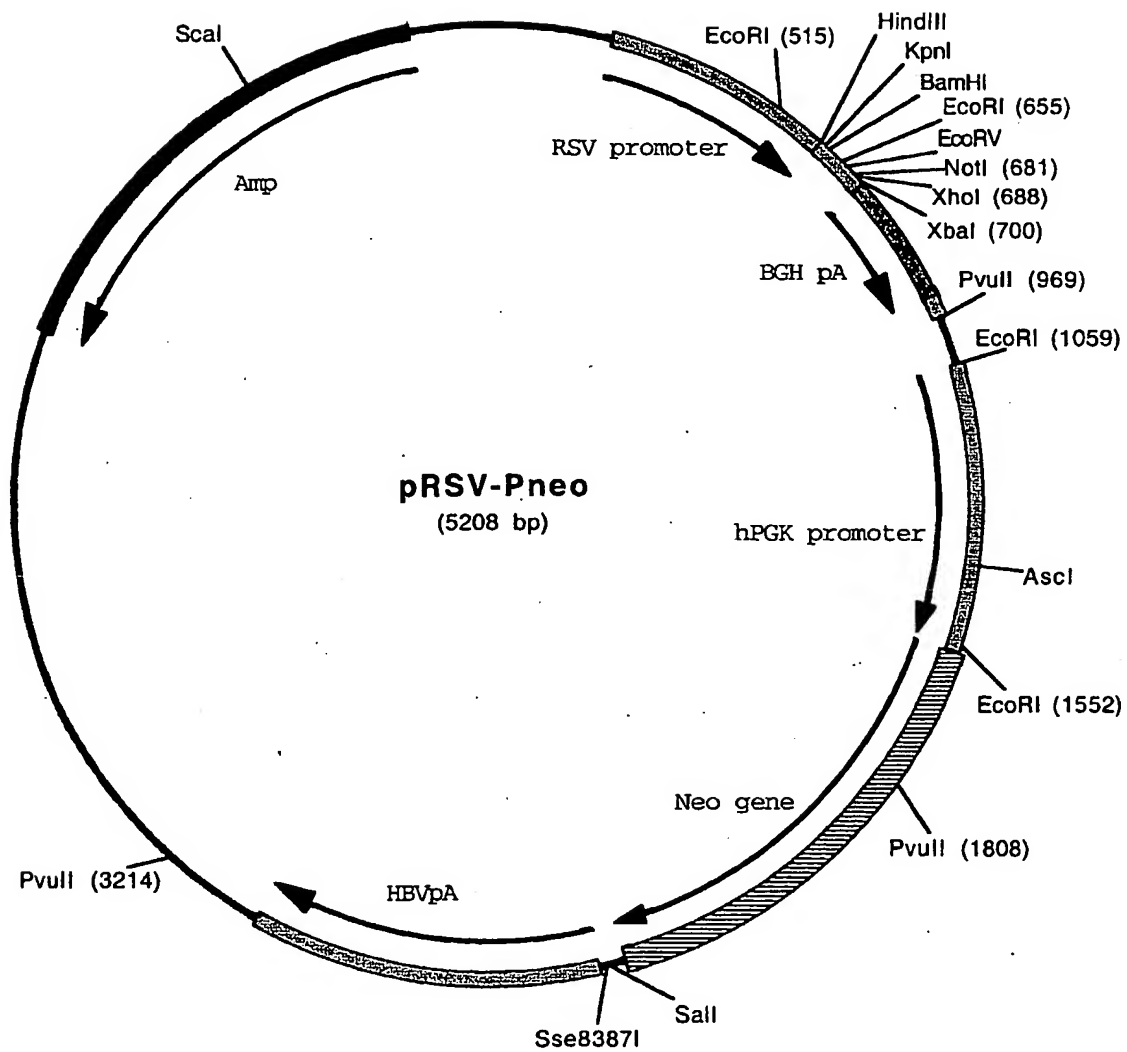
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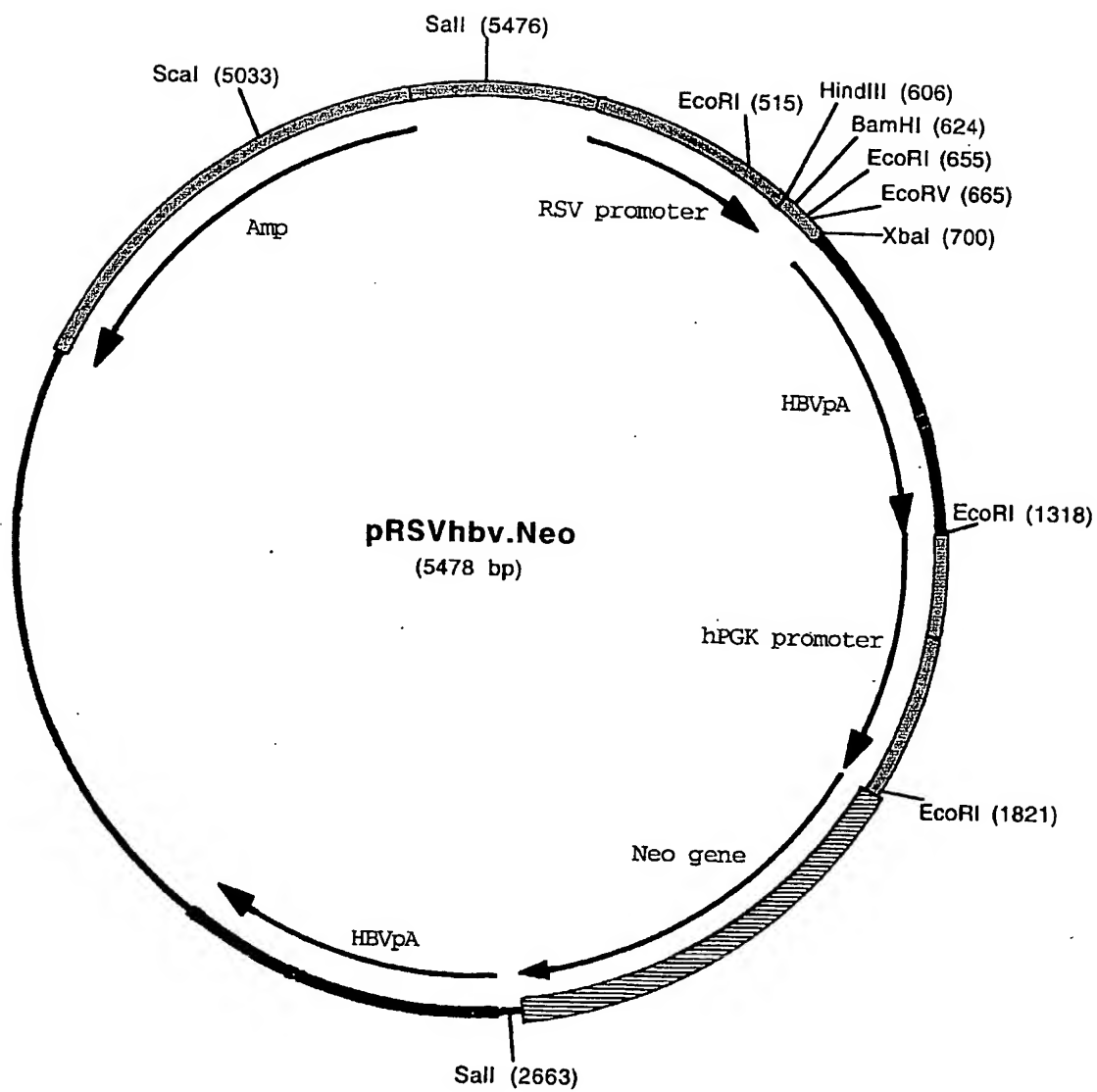
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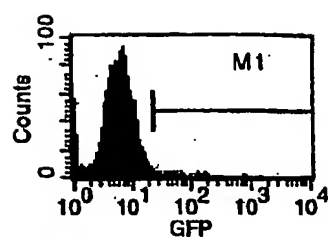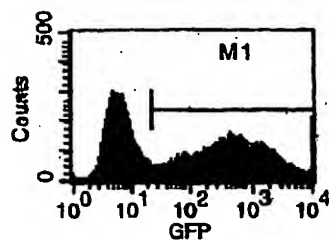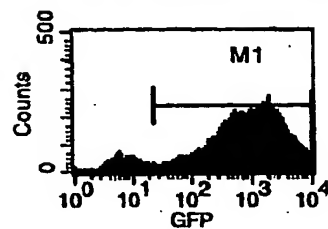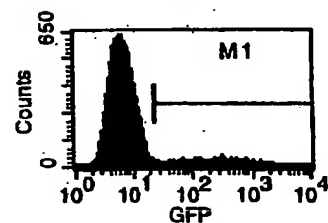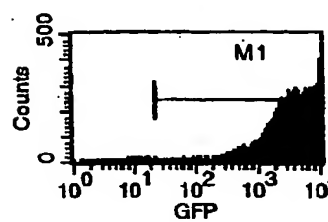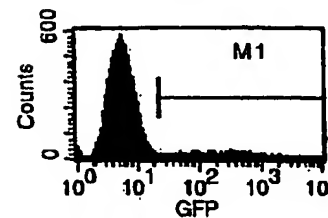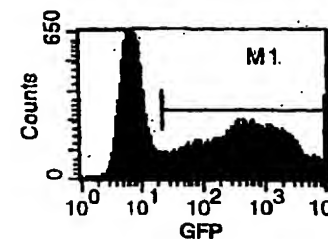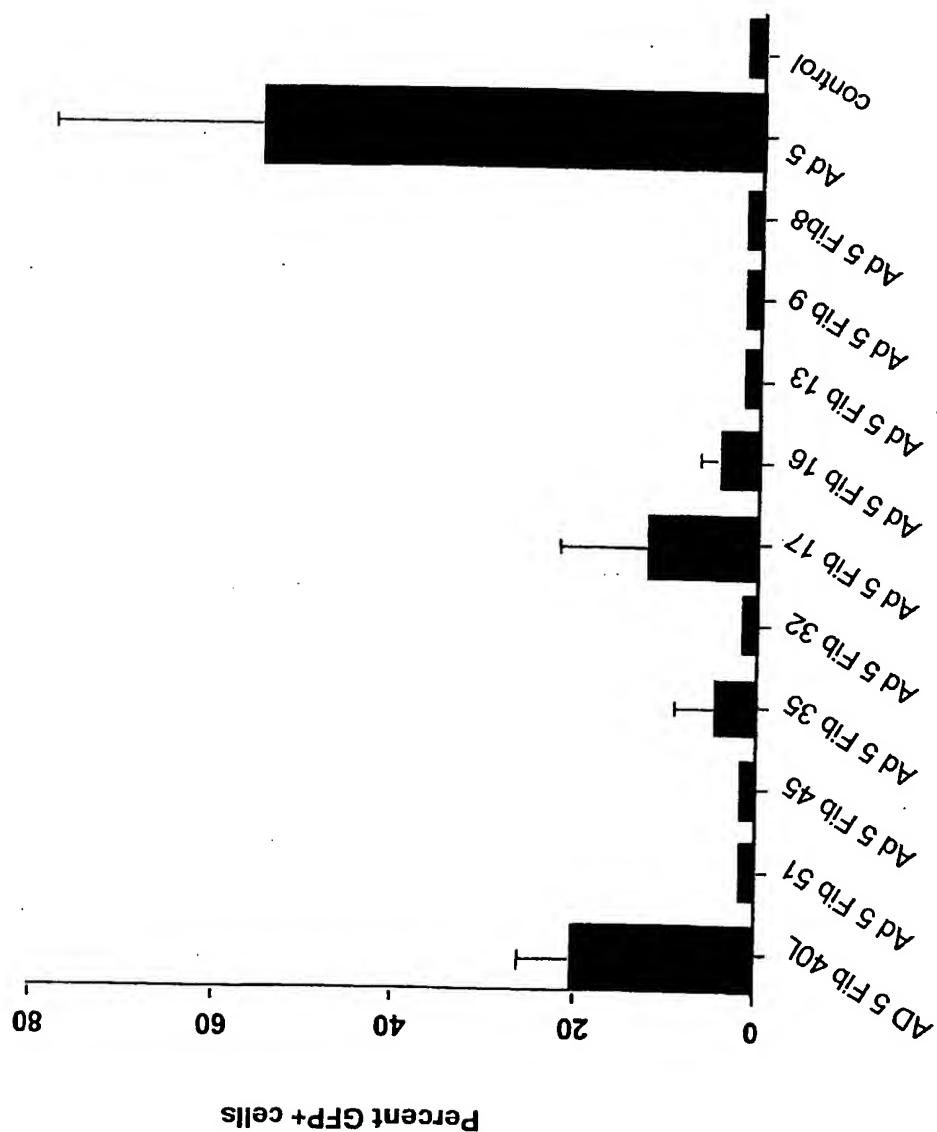
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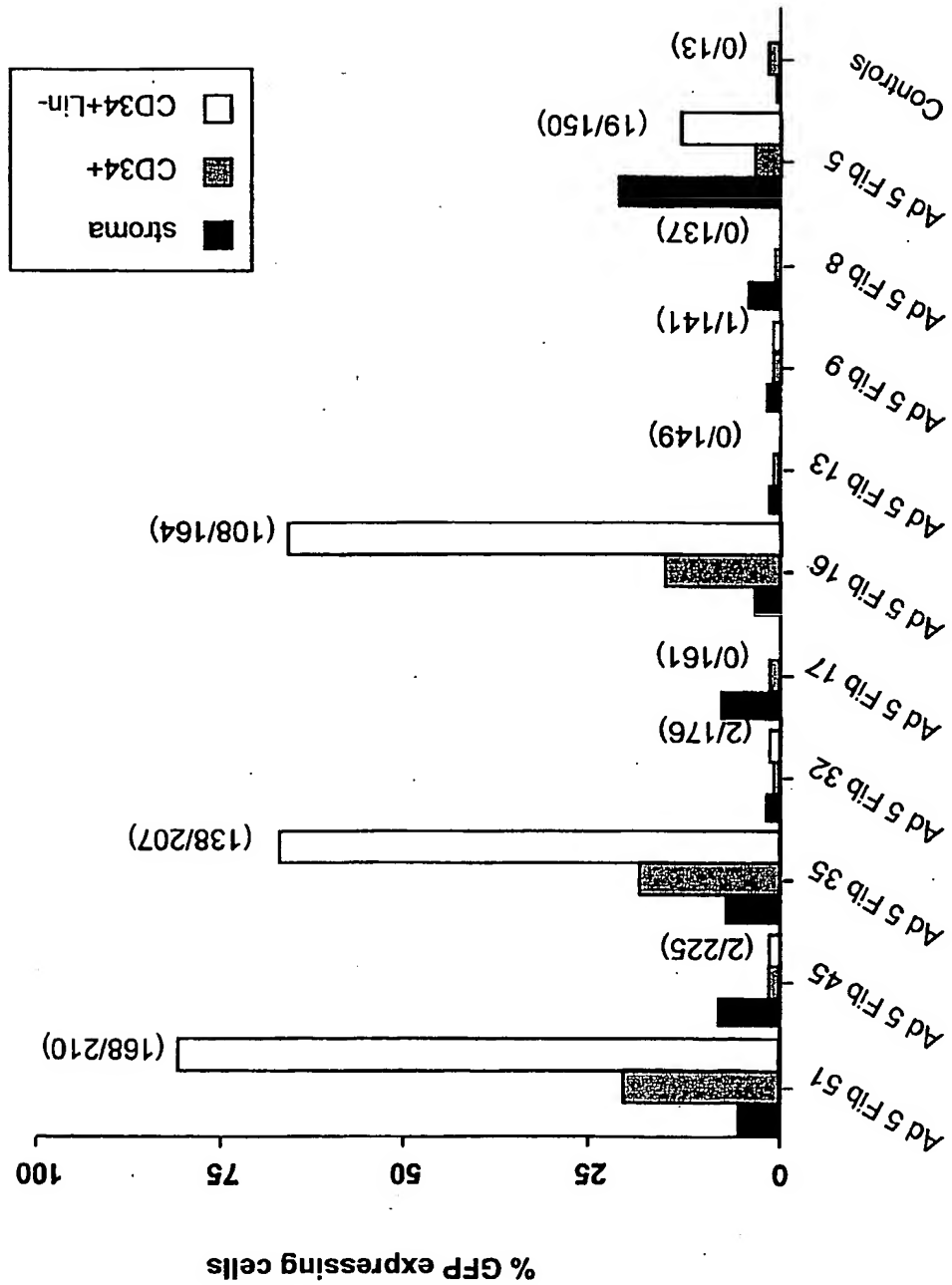
Figure 20

Figure 21

Figure 22a
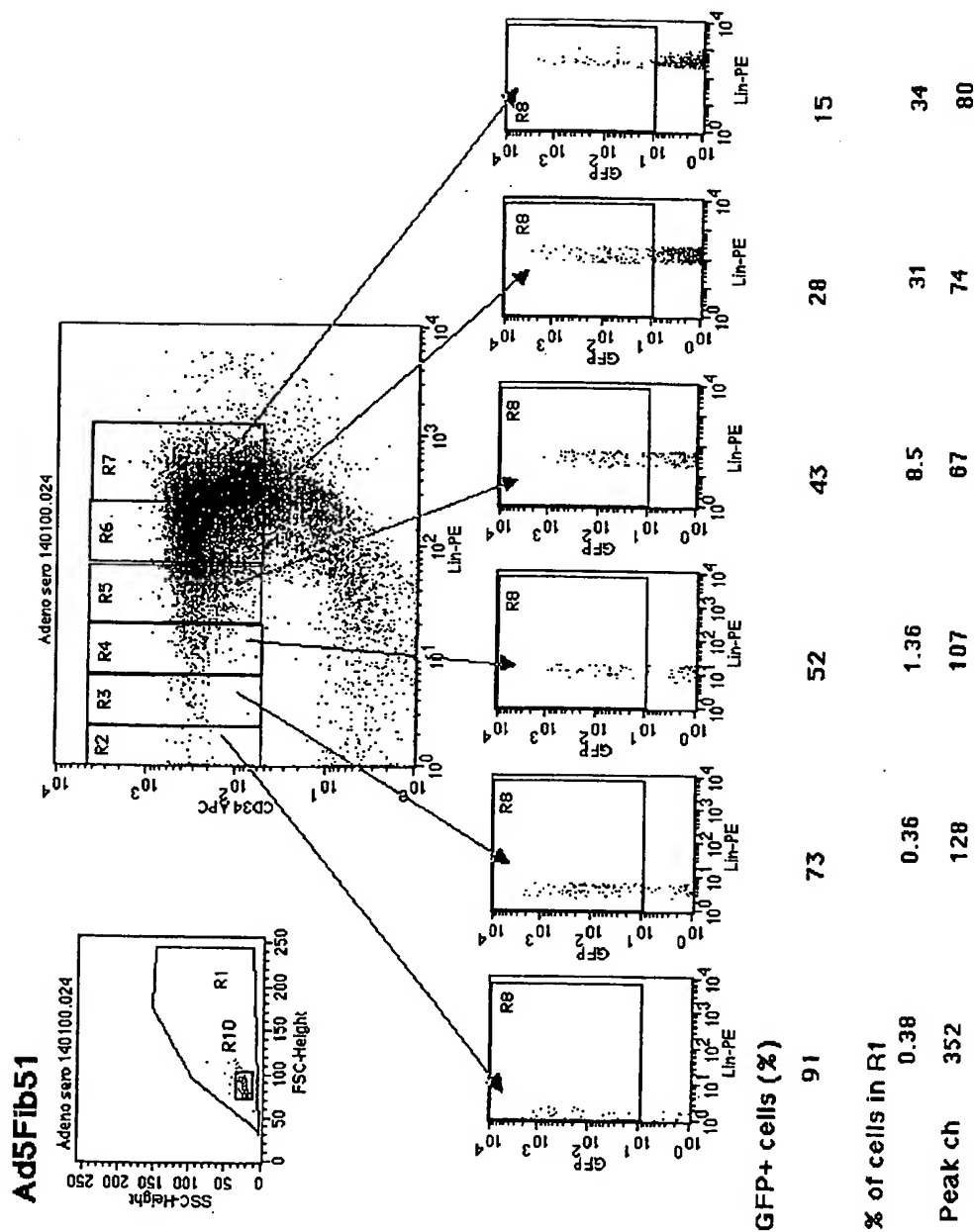
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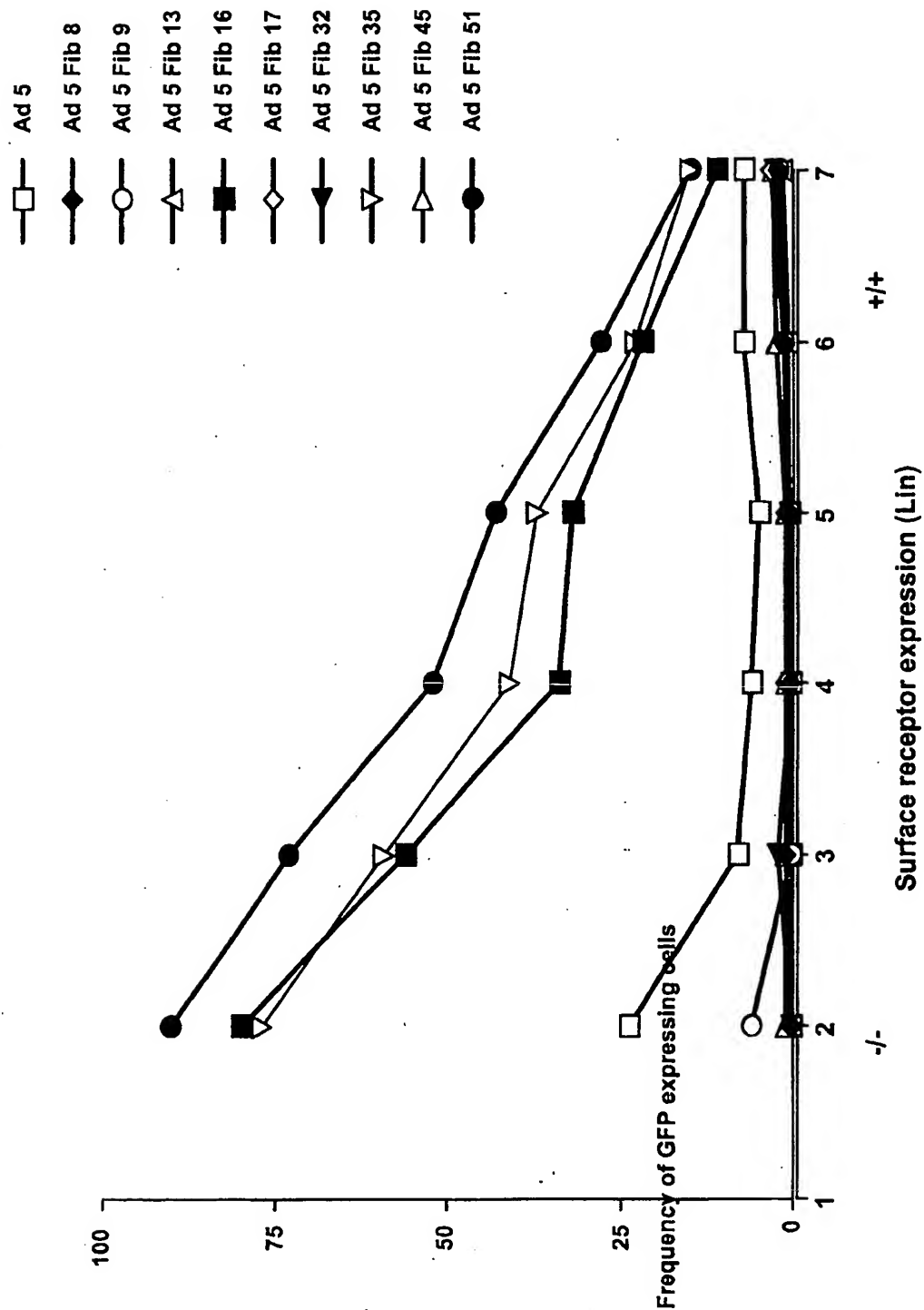
FIGURE 23

```
1   M L L Q M K R A R P S E D T F N P V Y P Y D T E T G P P T V P F L T P P F V S P   Fib 5 protein
1   M L L Q M K R A R P S E D T F N P V Y P Y E D E S S Q H - P F I N P G F I S S   Fib 16 protein
1   M L L Q M K R A R P S E D T F N P V Y P Y E D E S T S Q H - P F I N P G F I S P   Fib 35 protein
1   M L L Q M K R A R P S E D T F N P V Y P Y E D E S T S Q H - P F I N P G F I S P   Fib 51 protein

41  N G F Q E S P P G V L S L R L S E P L V T S N G M L A L K M G N G L S L D E A G   Fib 5 protein
40  N G F A Q S P D G V L T L K C V N P L T T A S G P L Q L K V G S S L T V D T I D   Fib 16 protein
40  N G F T Q S P D G V L T L K C L T P L T T G G S L Q L K V G G G L T V D D T D   Fib 35 protein
40  N G F T Q S P D G V L T L N C L T P L T T T G P L Q L K L V G G L I V D D T D   Fib 51 protein

81  N L T S Q N V T T V S P P L K K T K S N I N L E I S A P L T V T S E A L T V A A   Fib 5 protein
80  G S L E E N I T - A A A P L T K T N H S I G L L I G S G L Q T - - - - - - - - -   Fib 16 protein
80  G T L Q E N I R - A T A P I T K N N H S V E L S I G N G L E T - - - - - - - - -   Fib 35 protein
80  G T L Q E N I R - V T A P I T K N N H S V E L S I G N G L E T - - - - - - - - -   Fib 51 protein

121 A A P L M V A G N T L T M Q S Q A P L T V H D S K L S I A T Q G P L T V S E G K   Fib 5 protein
110 - - - - - - - - - - - - - - - - - K D K L C L S L E D G L V T K D D K   Fib 16 protein
110 - - - - - - - - - - - - - - - - - Q N N K L C - - - - - - - - - - - -   Fib 35 protein
110 - - - - - - - - - - - - - - - - - Q N N K L C - - - - - - - - - - - -   Fib 51 protein

161 L A L Q T S G P L T T T D S S T L T I T A S P P L T T A T G S L G I D L K E P I   Fib 5 protein
129 L G L - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -   Fib 16 protein
116 - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -   Fib 35 protein
116 - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -   Fib 51 protein

201 Y T Q N G K L G L K Y G A P L H V T D D L N T L T V A T G P G V T I N N T S L Q   Fib 5 protein
132 - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -   Fib 16 protein
116 - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -   Fib 35 protein
116 - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -   Fib 51 protein

241 T K V T G A L G F D S Q G N M Q L N V A G G L R I D S Q N R R L I L D V S Y P F   Fib 5 protein
132 - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -   Fib 16 protein
116 - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -   Fib 35 protein
116 - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -   Fib 51 protein

281 D A Q N Q L N L R L G Q G P L F I N S A H N L D I N Y N K G L Y L F T A S N N S   Fib 5 protein
132 - - - - - - - - S L G D G - - - - - - - - - - - - - - - - - - - - - - - - - - -   Fib 16 protein
116 - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -   Fib 35 protein
116 - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -   Fib 51 protein

321 K K L E V N L S T A K G L M F D A T A I A I N A G D G L E F G S P N A P N T N P   Fib 5 protein
137 - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -   Fib 16 protein
116 - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -   Fib 35 protein
116 - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -   Fib 51 protein

361 L K T K I G H G L E P D S N K A M V P K L G T G L S F D S T G A I T V G N K N N   Fib 5 protein
137 L L T K - - - - - - - N D V L C A K L G H G L V P D S - S N X - I T I E N N   Fib 16 protein
116 - - - - - - - - - - - A K L G N G L K F N N - G D I C I K D S I N   Fib 35 protein
116 - - - - - - - - - - - A K L G N G L K F N N - G D I C I K D S I N   Fib 51 protein

401 D K L T L W T T P A P S P N C R L - - - N A E K D A K L T L V L T K C G S Q I L   Fib 5 protein
166 - - - T L W T G A K P S A N C V I K E G E D S P D C K L T L V L V K N G G L I N   Fib 16 protein
137 - - - T L W T G I N P P P N C Q I V E N T N T N D G K L T L V L V K N G G L V N   Fib 35 protein
137 - - - T L W T G I K P P P N C Q I V E N T D T N D G K L T L V L V K N G G L V N   Fib 51 protein

438 A T V S V L A V K G S L A P I - S G T V Q S A H L I I R F D E N G V L L N N S P   Fib 5 protein
203 G Y I T L M G A S E Y T N T L P K N N Q V T I D V N L A F D N T G Q I I T Y L S   Fib 16 protein
174 G Y V S L V G V S D T V N Q M F T Q K T A N I Q L R L Y F D S S G N L L T E E S   Fib 35 protein
174 G Y V S L V G V S D T V N Q M F T Q K S A T I Q L R L Y F D S S G N L L T D E S   Fib 51 protein

477 L D P E Y W N F R N G D L T E G T A Y T N A V G F M P N L S A Y P K S H G K T A   Fib 5 protein
243 S L K S N L N F K D N Q N M A T G T I T S A R G F M P S T T A Y P F I - - - T Y   Fib 16 protein
214 D L K I P L K N K S S T A - T S E T V A S S K A F M P S T T A Y P F N - - - T T   Fib 35 protein
214 N L K I P L K N K S S T A - T S E A T S S K A F M P S T T A Y P F N - - - T T   Fib 51 protein

517 K S N I V S Q V Y L N G D - - K T K - - - - - P V T L T I T L N G T Q E T G   Fib 5 protein
280 A T E T L N E D Y I Y G E C Y Y K S T - N G T L F P L K V T V T L N R R M L A S   Fib 16 protein
250 T R D S - - E N Y I H G I C Y Y M T S Y D R S L F P L N I S I M L N S R M I S S   Fib 35 protein
250 T R D S - - E N Y I H G I C Y Y M T S Y D R S L V P L N I S I M L N S R T I S S   Fib 51 protein

548 D T T P S A Y S M S F S W D W S G H N Y I N E - - - I F A T S S Y T F S Y I A Q   Fib 5 protein
319 G M - - - A Y A M N F S W S L N A E E A P E T T E V T L I T S P F F F S Y I R E   Fib 16 protein
288 N V - - - A Y A I Q F E W N L N A S E S P E S N I M T L T T S P F F F S Y I T E   Fib 35 protein
288 N V - - - A Y A I Q F E W N L N A K E S P E S N I A T L T T S P F F F S Y I I E   Fib 51 protein

585 E .                                                                  Fib 5 protein
356 D .                                                                  Fib 16 protein
325 D .                                                                  Fib 35 protein
325 D .                                                                  Fib 51 protein
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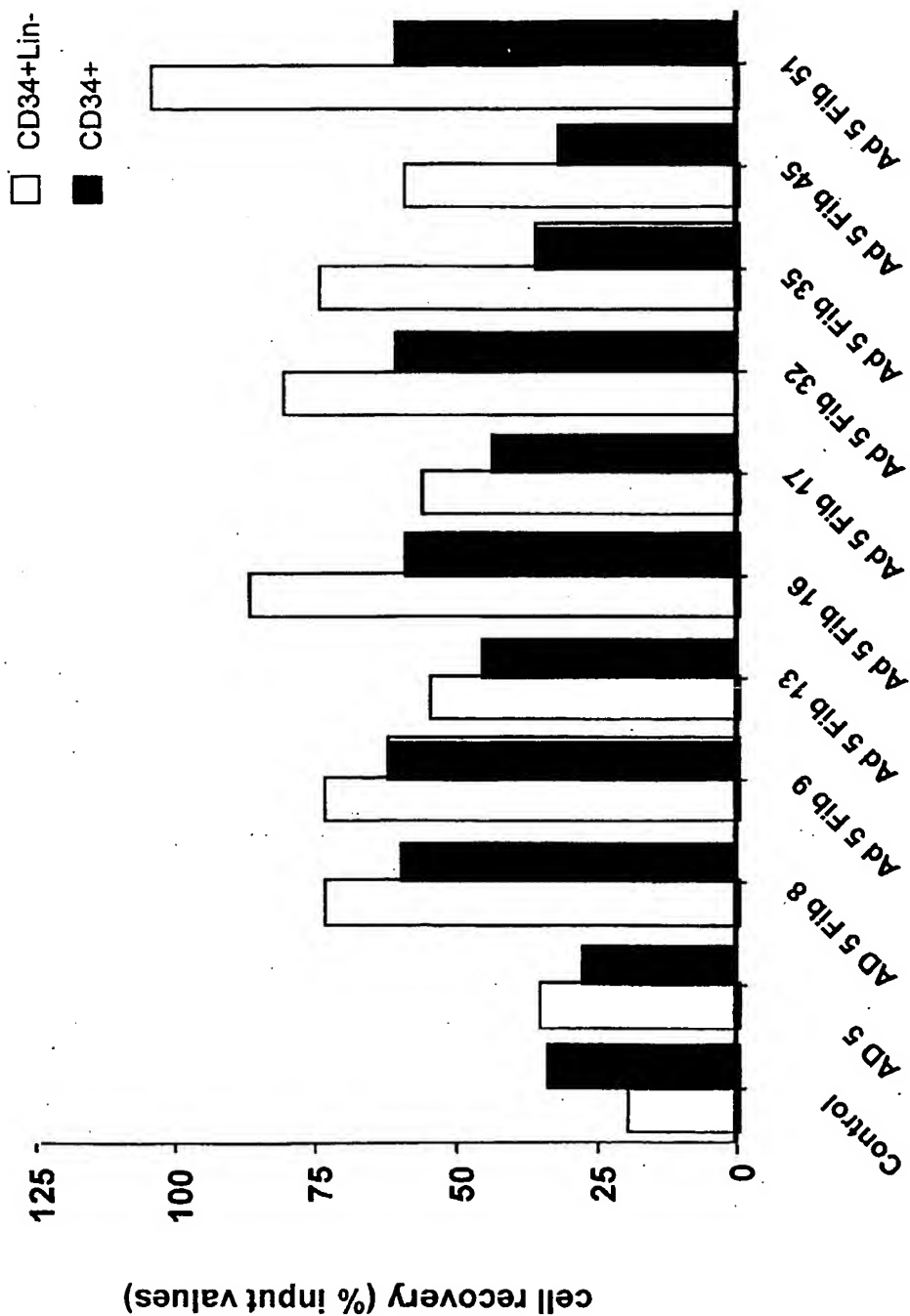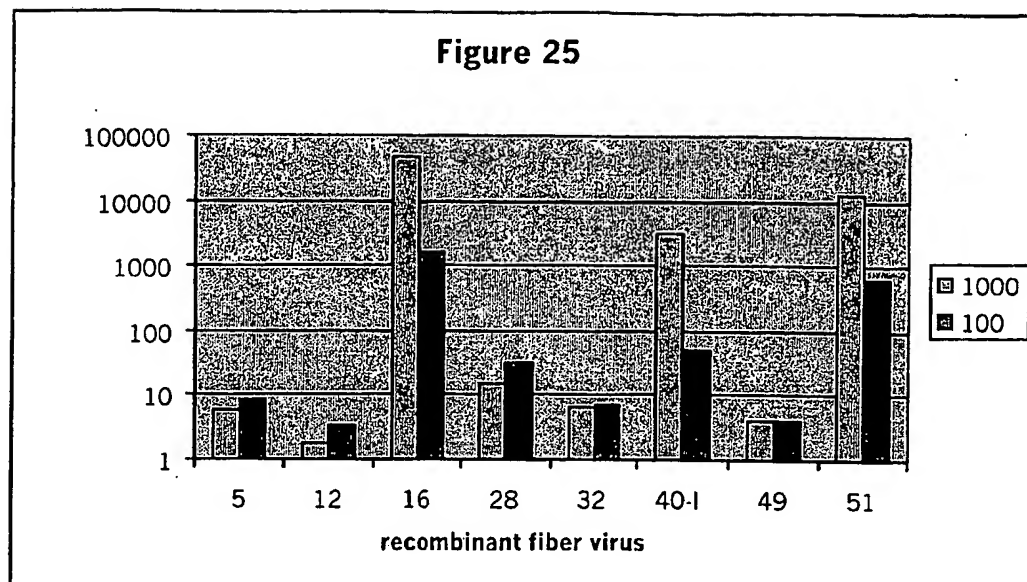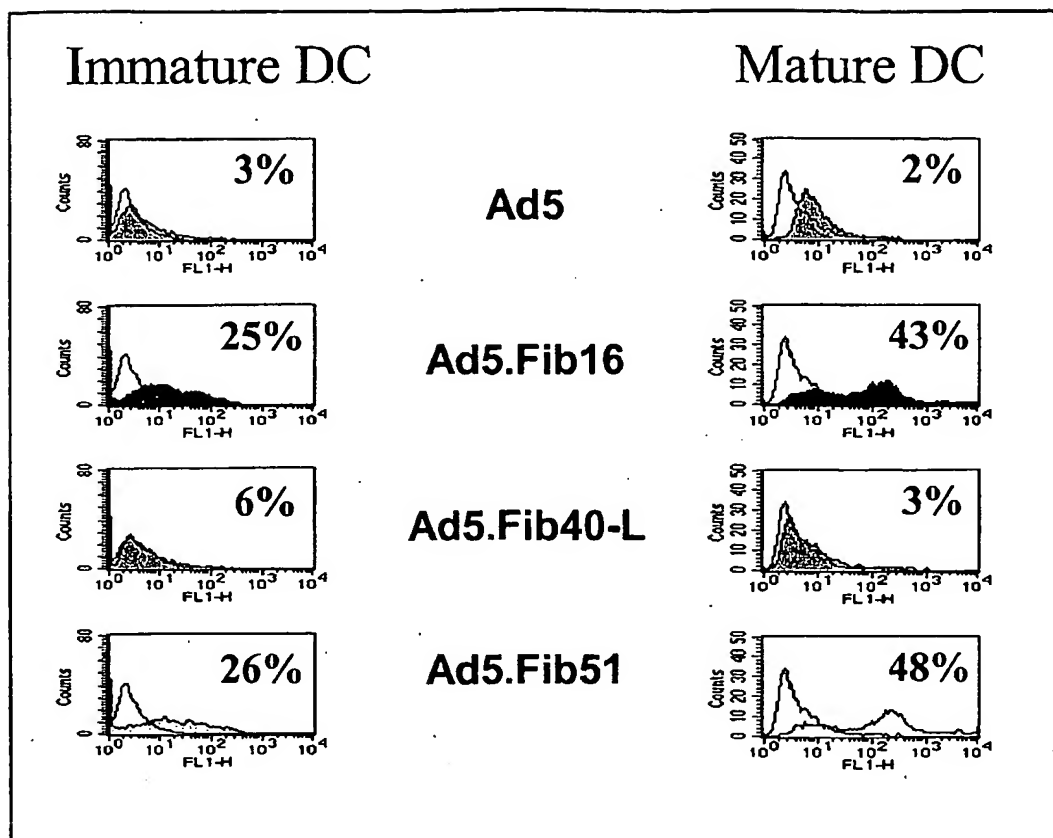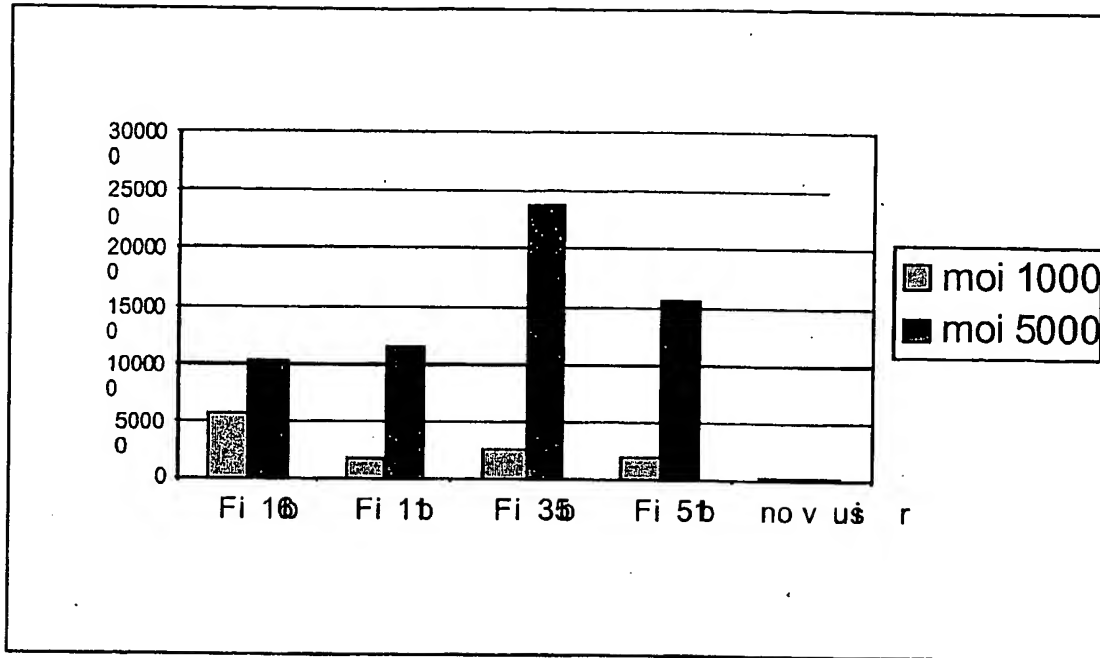
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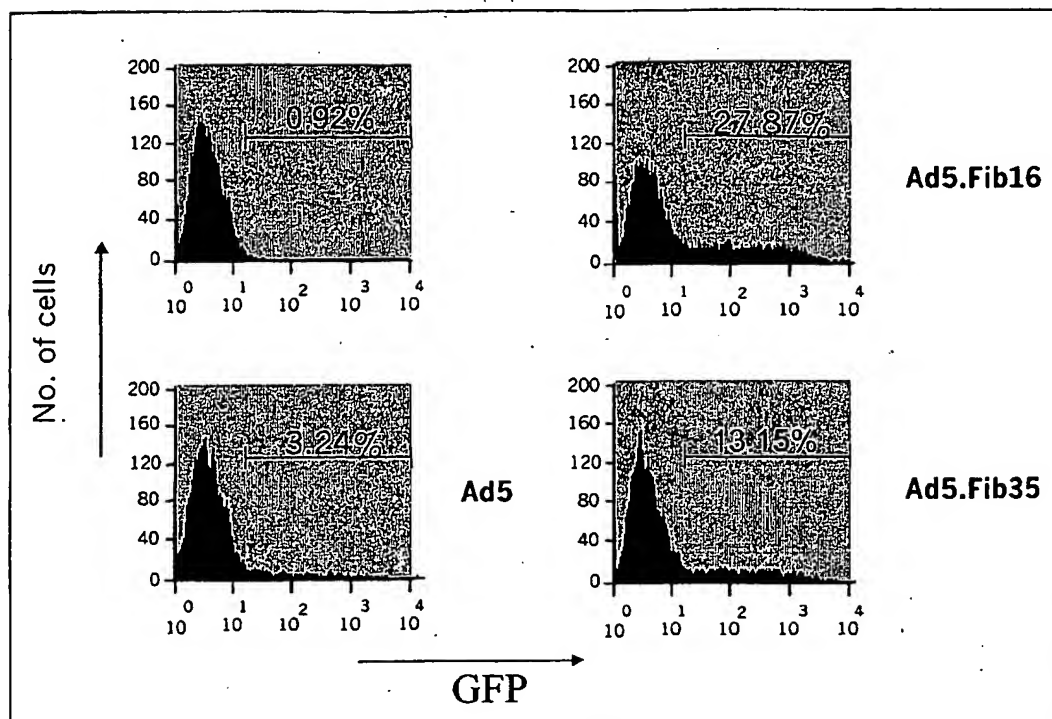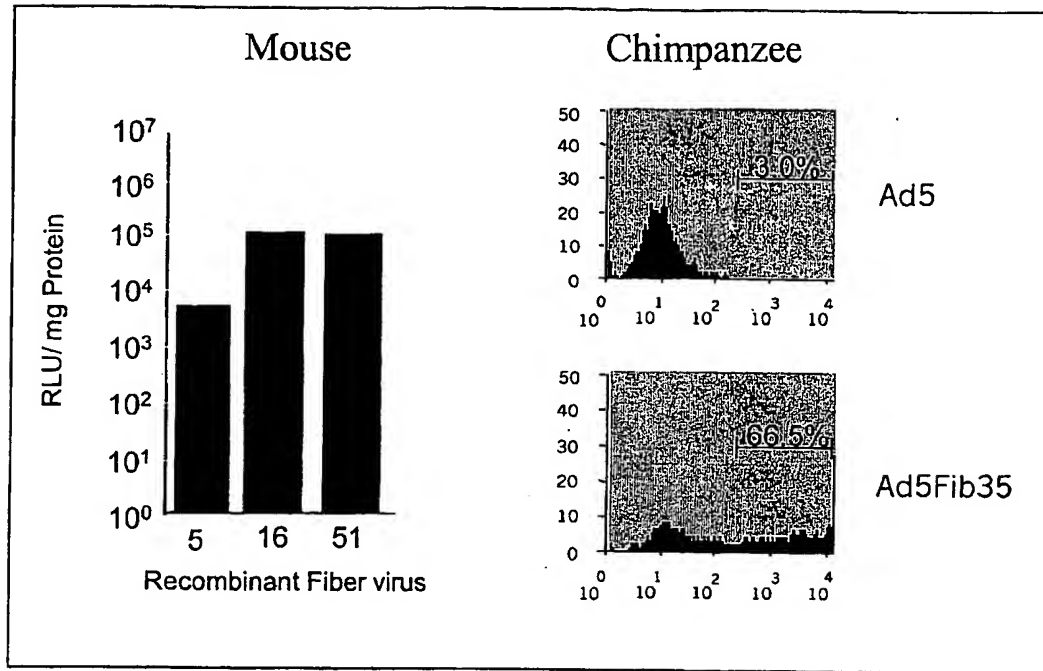Figure 24

Figure 25

Figure 26

Figure 27

Figure 28

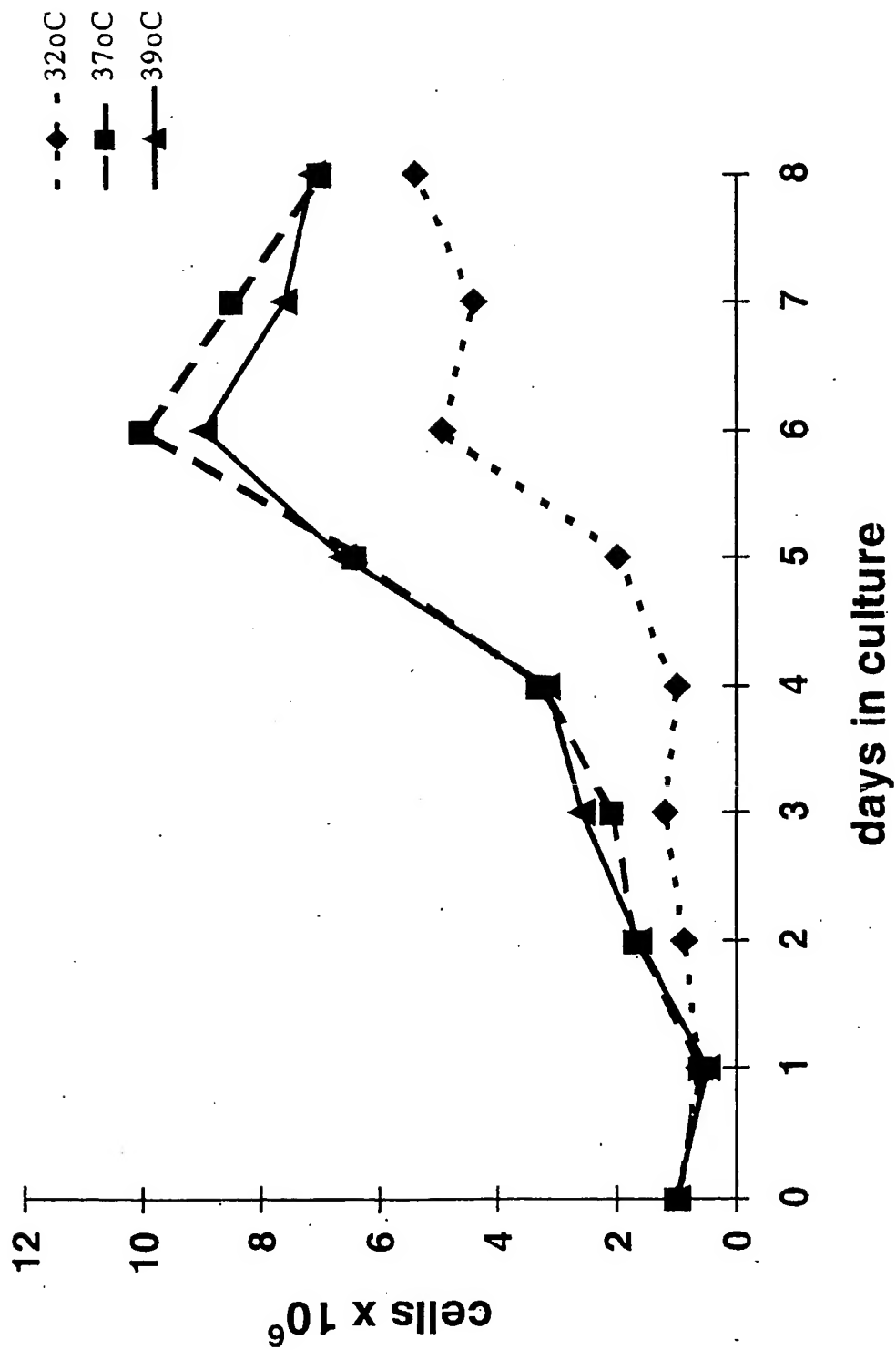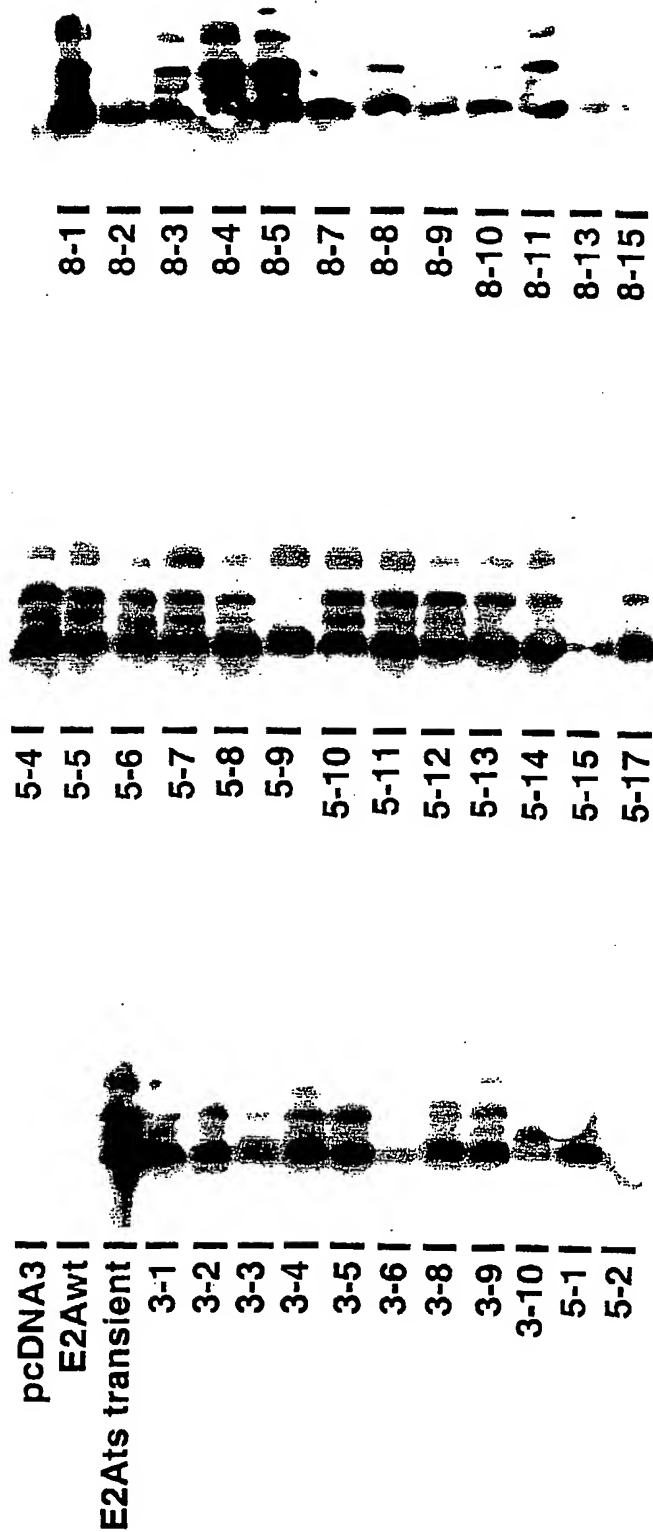# Figure 29

Figure 30 Temperature dependent growth of PER.C6

Figure 31　　DBP levels in PER.C6 cells transfected with pcDNA3, pcDNA3wtE2A or pcDNA3ts125E2A

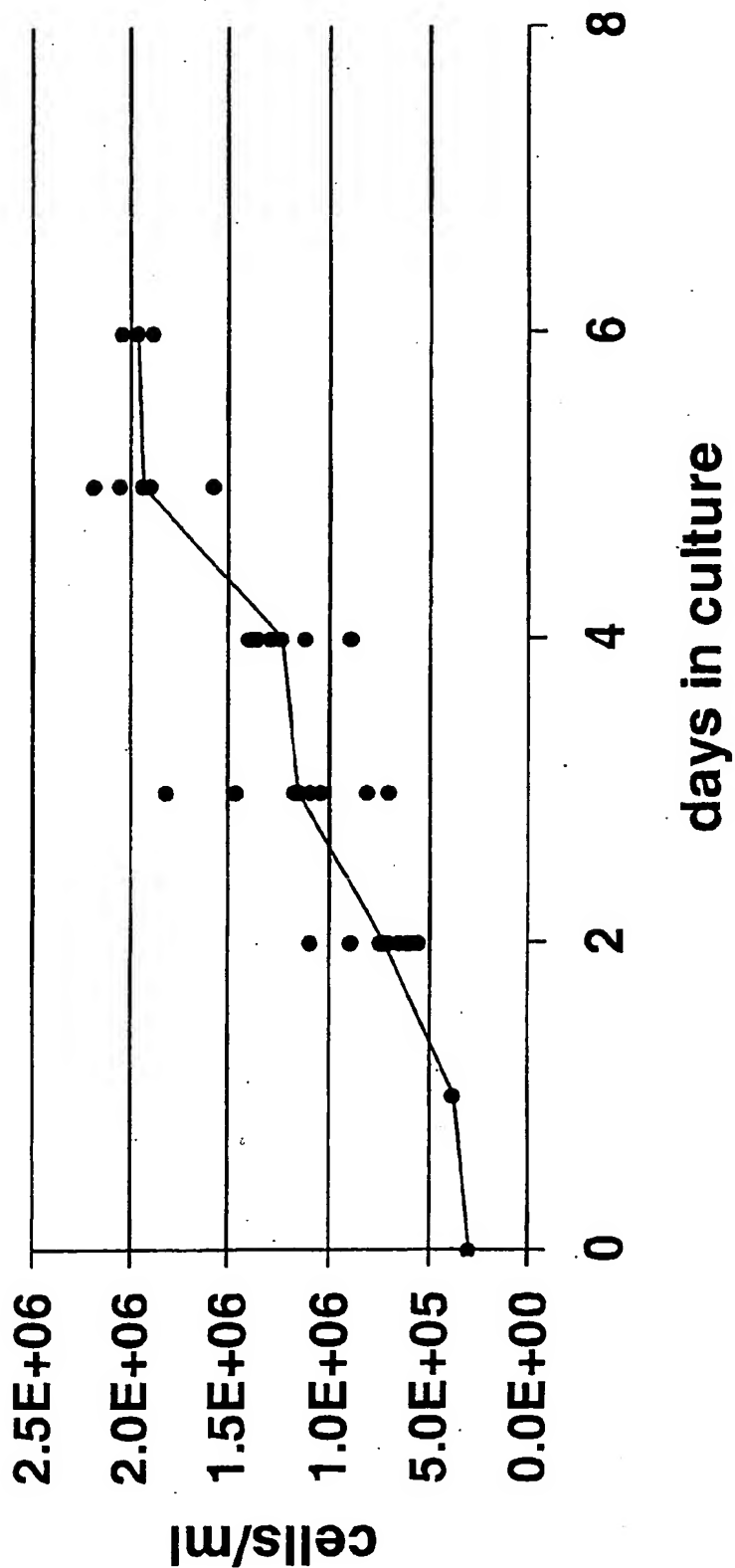Figure 32   Suspension growth of PER.C6ts125E2A C5-9
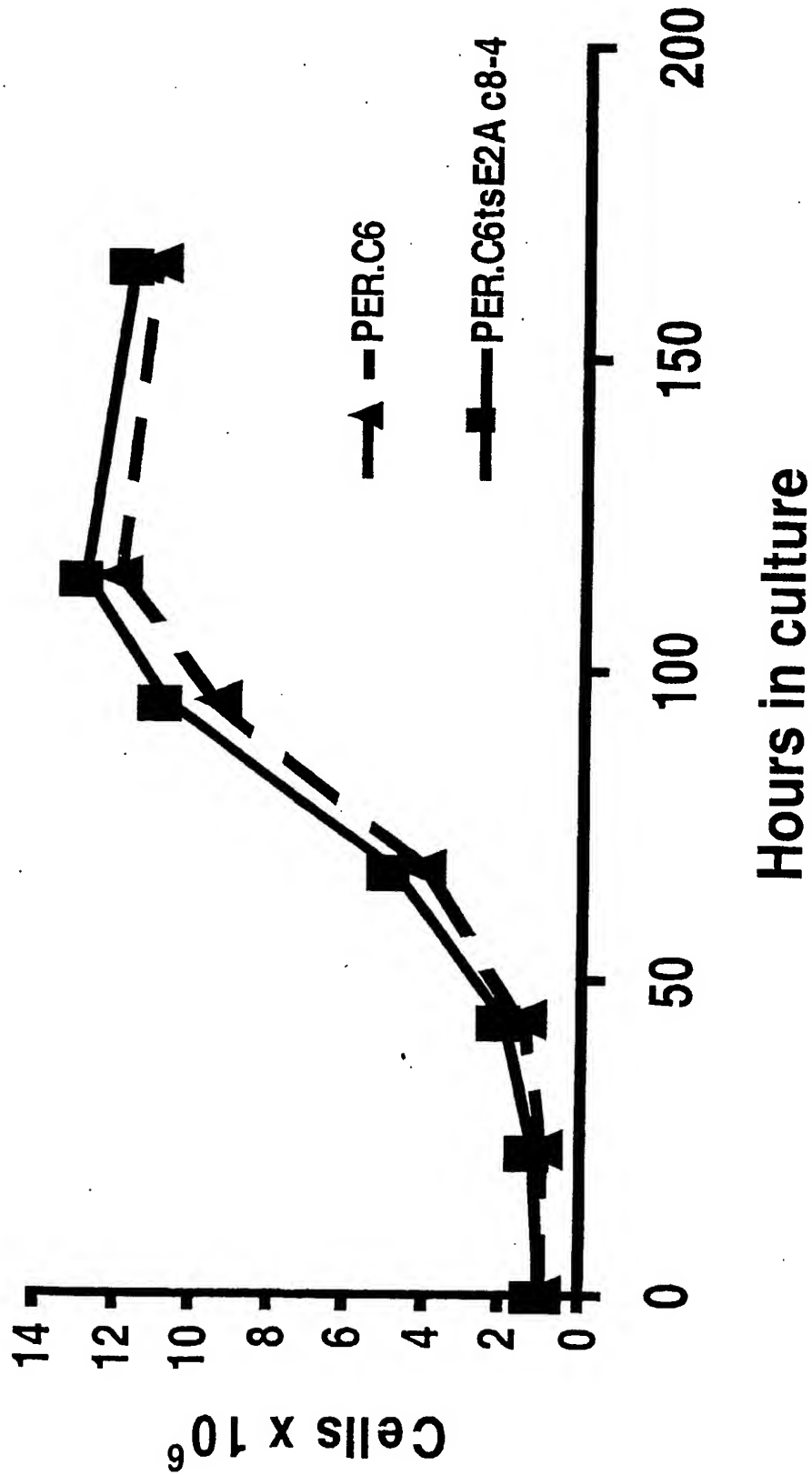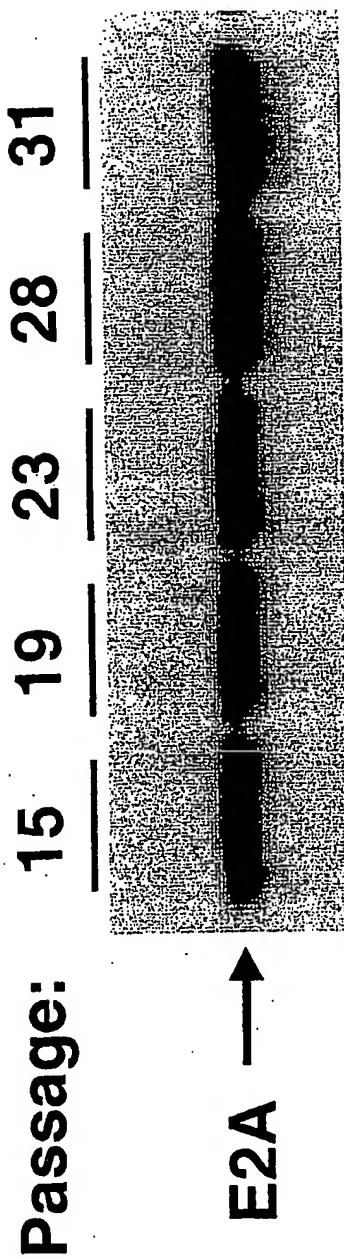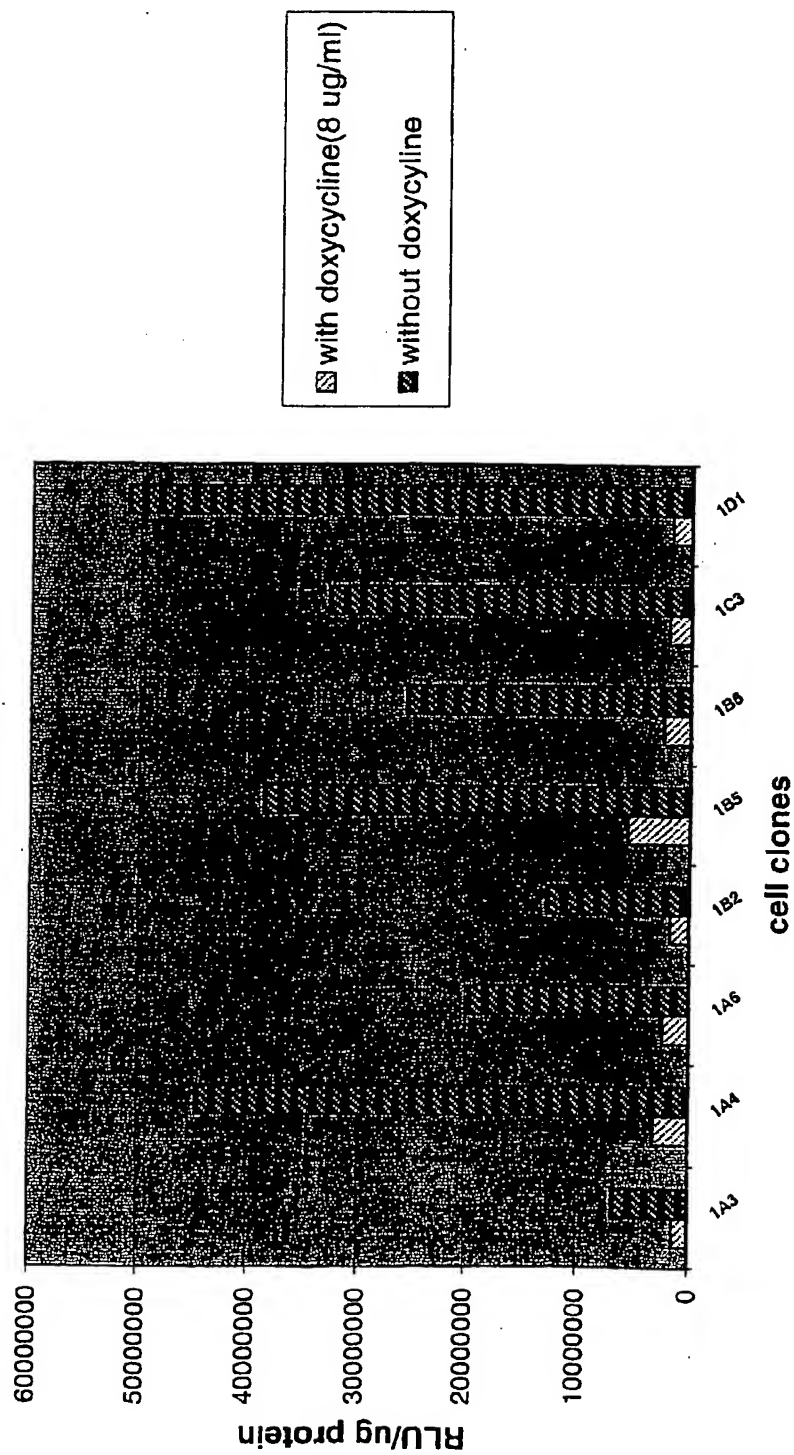
Figure 33 Growth curve PER.C6 and PER.C6tsE2A
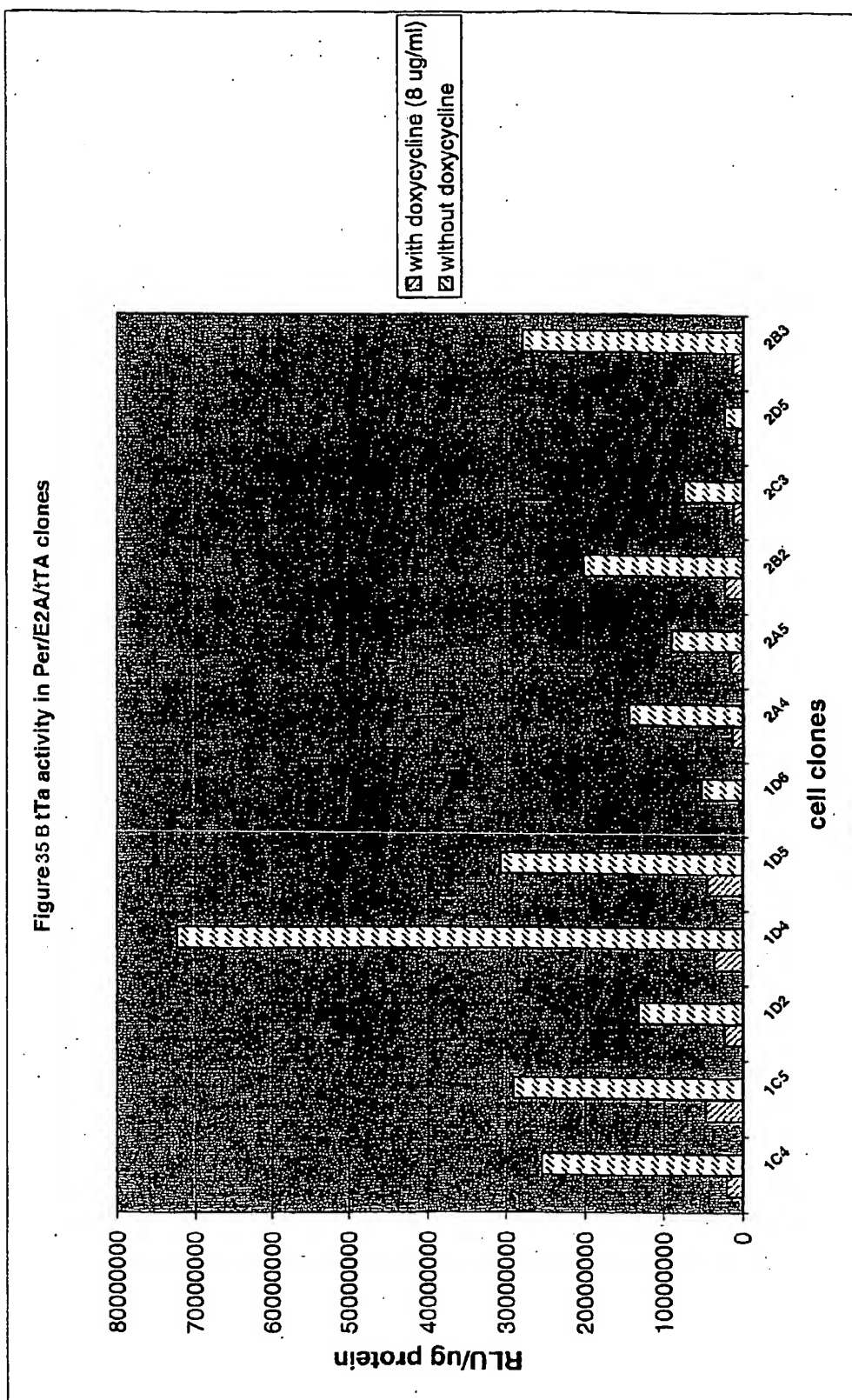
Figure 34   Stability of PER.C6ts125E2A

Figure 35A tTA activity in Per.C6/tTA clones

Figure 35 B tTa activity in Per/E2A/tTA clones

INTERNATIONAL SEARCH REPORT

International Application No

PCT/NL 00/00325

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7   C12N15/86   C12N15/34   C12N5/10   A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7   C12N   A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the International search (name of data base and, where practical, search terms used)

BIOSIS, EMBASE, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BASLER C. F. ET AL.: "Sequence of the immunoregulatory early region 3 and flanking sequences of adenovirus type 35" GENE, vol. 170, no. 2, 8 May 1996 (1996-05-08), page 249-254 XP004042835 ISSN: 0378-1119 cited in the application	8,9
Y	the whole document	1-3,5-7, 10-19, 21,22

X	WO 96 12030 A (RHONE POULENC RORER SA ;LEE MARTIN (FR); PERRICAUDET MICHEL (FR)) 25 April 1996 (1996-04-25)	23
Y	the whole document	1-3,5-7, 10-19, 21,22

	-/--	

[X] Further documents are listed in the continuation of box C.   [X] Patent family members are listed in annex.

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the International filing date but later than the priority date claimed

"T" later document published after the International filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"&" document member of the same patent family

Date of the actual completion of the International search	Date of mailing of the international search report
24 August 2000	07/09/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340–2040, Tx. 31 651 epo nl, Fax: (+31-70) 340–3016	Mandl, B

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category°	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	FALLAUX F. J. ET AL.: "New. helper cells and matched early region 1-deleted adenovirus vectors prevent generation of replication-competent adenoviruses" HUMAN GENE THERAPY, vol. 9, no. 13, 1 September 1998 (1998-09-01), pages 1909-1917, XP002111678 ISSN: 1043-0342 the whole document ---	14
Y	WO 96 13598 A (UNIV PENNSYLVANIA ;WILSON JAMES M (US); KELLEY WILLIAM M (US); FIS) 9 May 1996 (1996-05-09) the whole document ---	18,19
X	FLOMENBERG P. R. ET AL.: "Sequence and genetic organization of Adenovirus Type 35 early region 3." JOURNAL OF VIROLOGY, vol. 62, no. 11, 1988, pages 4431-4437, XP002119500 cited in the application the whole document ---	8,9
X	BASLER C.F. ET AL.: "Subgroup B adenovirus type 35 early region 3 mRNAs differ from those of the subgroup C adenoviruses." VIROLOGY, vol. 215, 1996, pages 165-177, XP002119501 the whole document ---	8,9
A	LEE M. G. ET AL.: "THE CONSTITUTIVE EXPRESSION OF THE IMMUNOMODULATORY GP 19K PROTEIN IN E1-, E3- ADENOVIRAL VECTORS STRONGLY REDUCES THE HOST CYTOTOXIC T CELL RESPONSE AGAINST THE VECTOR" GENE THERAPY, vol. 2, no. 4, 1 June 1995 (1995-06-01), pages 256-262, XP000561548 ISSN: 0969-7128 the whole document ---	1-23
A	WO 97 12986 A (CORNELL RES FOUNDATION INC) 10 April 1997 (1997-04-10) page 11, line 3 - line 35 page 13, line 1 - line 30 ---	1-23
A	WO 98 22609 A (ARMENTANO DONNA E ;GREGORY RICHARD J (US); GENZYME CORP (US); SMIT) 28 May 1998 (1998-05-28). the whole document ---	1-23

-/--

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	GALL J. ET AL.: "ADENOVIRUS TYPE 5 AND 7 CAPSID CHIMERA: FIBER REPLACEMENT ALTERS RECEPTOR TROPISM WITHOUT AFFECTING PRIMARY IMMUNE NEUTRALIZATION EPITOPES" JOURNAL OF VIROLOGY, vol. 70, no. 4, 1 April 1996 (1996-04-01), pages 2116-2123, XP002050655 ISSN: 0022-538X the whole document ---	1-23
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